In Vitro Studies on AMPA Receptor-Mediated Motor Neuron Death: Relevance for Amyotrophic Lateral Sclerosis

Thesis

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In vitro studies on AMPA receptor - mediated motor neuron death: relevance for Amyotrophic Lateral Sclerosis

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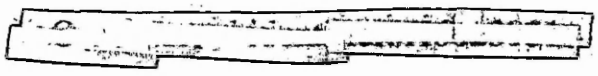

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a neurological disease mainly characterized by progressive motor neuron degeneration and muscle atrophy that lead to premature death. It is not yet fully understood in terms of etiology and, as a consequence, it is still orphan of cures. This project was aimed at studying some of the main reliable causal events leading to motor neuron degeneration in ALS, i. e. AMPA receptor (AMPAR)-dependent excitotoxicity, neuroinflammation and intracellular protein aggregation.

1) We investigated the intracellular mechanisms that are induced in motor neurons by AMPAR-mediated excitotoxicity, demonstrating that different death pathways were activated depending on the intensity of the initial stimulus to the receptor. Low AMPAR agonist concentrations induce, indeed, the typical intracellular events of the apoptotic pathway, while higher concentrations trigger to non-apoptotic motor neuron death.

2) We analysed the intracellular effect of mediators of the inflammatory signalling, i.e. TNF-α and IL-8, and their interaction with the AMPAR-dependent excitotoxic pathway. We demonstrated that IL-8-induced motor neuron death is specifically mediated by the CXCR2 chemokine receptor. TNF-α exerts both neurotoxicity and neuroprotection against AMPAR-mediated cell death. The presence of mature and active glial population is determinant in mediating TNF-α effect.

3) We studied the effect of intracellular α-synuclein accumulation in motor neurons, revealing a dual concentration-dependent effect since micromolar protein concentrations are neurotoxic, while nanomolar concentrations induce neuroprotective effect against oxidative stress.

In light of such results, we tested the effectiveness of potentially neuroprotective drugs which could interfere with the intracellular death mechanisms of motor neurons. We found that Erythropoietin (EPO) and different non-erythropoietic EPO derivatives (CEPO, ASIALOEPO and HBP) have specific neuroprotective properties against the

ABSTRACT

apoptotic AMPAR-dependent death pathway. Reparixin, an orally active chemokine receptor (CXCR1/2) inhibitor was successfully tested against the IL-8-dependent motor neuron death.

All together these results add further useful information to define the complex ALS etiology and provide interesting pharmacological approaches which could be relevant for the treatment of the pathology.

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LIST OF ABBREVIATIONS

α-syn, α-synuclein

-/- mice, CXCR2 deficient mice

$[Ca^{2+}]_i$, intracellular calcium concentration

+/- mice, heterozygous mice for CXCR2

+/+ mice, wild type mice expressing CXCR2

Ab, antibody

AIFs, Apoptotic Initiating Factors

ALS FRS, Amyotrophic Lateral Sclerosis Functional Rating Scale

ALS, Amyotrophic Lateral Sclerosis

AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate

AMPAR, AMPA Receptor

AraC, 1-β-D-arabino-furanosyl-cytosine

ATP, adenosine-5'-triphosphate

BDNF, Brain-Derived Neurotrophic Factor

BSA, Bovine Serum Albumin

CD131, common IL-3/IL-5/GM-CSF receptor

CEPO, carbamylated-EPO

CNS, Central Nervous System

CNTF, Ciliary Neurotrophic Factor

CSF, Cerebro Spinal Fluid

CTR, control

CXCR2, receptor for ELR^+ CXC chemokines (i.e. IL-8, MIP-2)

DAB, 3,3'-Diaminobenzidine

DF1726A, reparixin analogue

DIV, Days In Vitro

DMSO, Dimethyl Sulfoxide

E.C.50, Effective Concentration causing 50% effect (i.e. 50% cell death)

EAAT2, Excitatory Amino-Acid Transporter 2, glial glutamate transporter

EAE, Experimental Autoimmune Encephalomyelitis

EDTA, ethylenediaminetetraacetic acid

EPO, Erythropoietin

fALS, familial Amyotrophic Lateral Sclerosis

FBS, Fetal Bovine Serum

LIST OF ABBREVIATIONS

FCS, Fetal Calf Serum

GFAP, Anti-Glial Fibrillary Acid Protein

GluR, Glutamate Receptor

h, hour

HBP, helix B peptide

HIF-1, Hypoxia-Inducible Factor-1

I.C.50, Inhibitory Concentration causing 50% inhibition of the effect of a compound

IGF-1, Insulin-like nerve Growth Factor 1

IL-8, Interleukin-8

KA, Kainic Acid

min, minute

MIP-2, (or GROb/CXCL2) CXCR2 agonist

mSOD1, mutant SOD1

NBQX, (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), AMPAR antagonist

NF-H heavy neurofilaments

NF-L, light neurofilaments

NF-M, medium neurofilaments

NGS, Normal Goat Serum

NMDA, N-methyl-D-aspartate

NMDAR, NMDA Receptor

PBS, Phosphate Buffered Saline

PI, Propidium Iodide

ROS, Reactive Oxygen Species

RT, Room Temperature

S.D., Standard Deviation

sALS, sporadic Amyotrophic Lateral Sclerosis

SMA, Spino Muscular Atrophy

SOD1, copper/zinc superoxide dismutase

TBS, Tris-Buffered Saline

TNF, Tumor Necrosis Factor

TNFR, TNF-α Receptor

VEGF, Vascular Endothelial Growth Factor

Vps54, Vacuolar–vesicular Protein Sorting

LIST OF ABBREVIATIONS

Special characters

Å, Armstrong = 10^{-10} m

Da, Dalton = 1,6 x 10^{-24} g

kDa, Kilodalton = 1000 dalton

$\Delta\Psi M$, mitochondrial transmembrane potential

μM, micromolar

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INTRODUCTION

Chapter 1 - ALS

1.1 History

Amyotrophic lateral sclerosis is a severe neurological disease associated with degeneration and loss of motor neurons at different levels of the motor system. "Amyotrophic" refers to muscle weakness, atrophy and fasciculation that account for the effect on motor neurons. "Lateral sclerosis" relates to the rigidity of the lateral columns of the spinal cord in autopsy specimens due to gliosis of the cortico-spinal tracts (Rowland & Shneider 2001). The French neurobiologist Jean-Martin Charcot first clearly identified the disease (also known as "Maladie de Charcot") in 1869 (Charcot 1869). ALS is also colloquially known in the United States as Lou Gehrig's disease, named after the New York Yankee baseball player who developed the disease in the 1930s. Many other well known individuals have been affected by the pathology during recent years, including professional Italian soccer players who have seen a ten-fold increased incidence of ALS (Belli & Vanacore 2005). The Nobel-prize-winning astrophysicist Stephen Hawking, suffering from an unusually slowly progressing form of the disease, is an example demonstrating how selective the neuronal loss can be. He has no cognitive impairment, but an almost complete paralysis of his arms, legs and the muscles necessary for speech (schematic summary of the main features of the disease reported in Fig. 1.1). On the other hand, ALS is a very heterogeneous disease displaying a huge complexity in terms of genetic, biochemical and clinical features. Indeed, despite developments in histopathological techniques, the introduction of clinical neurophysiology, and the use of neuroimaging techniques, the diagnosis of ALS still requires the skills of an experienced neurologist with techniques that were available at the turn of the nineteenth century. Pathogenic mechanisms of the disease are still unknown and a variety of potentially toxic events could account for the onset of the

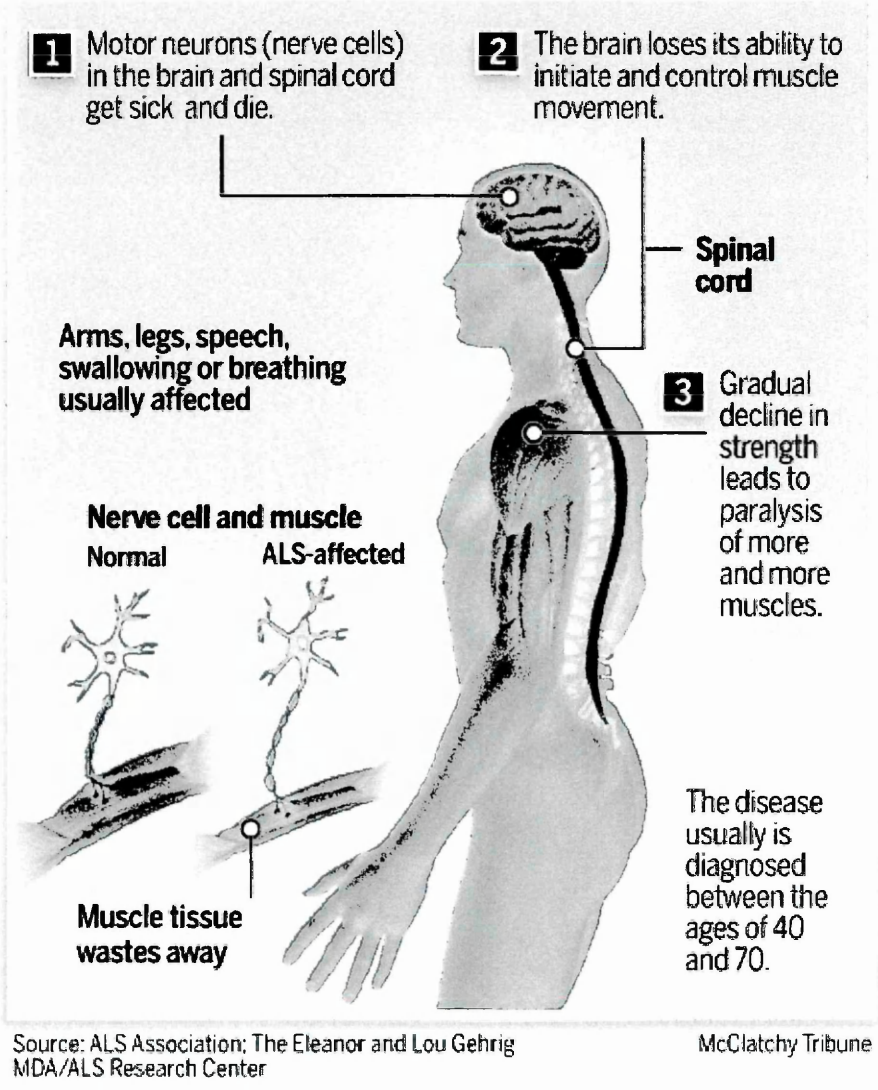


Fig. 1.1 Schematic recapitulation of the main events involved in ALS.

disease. As a consequence of this complexity and heterogeneity, potential biomarkers have never been assessed and therapeutic strategies are hard to devise. In fact, although numerous compounds have been tested in clinical trials, as yet only riluzole has been proven effective for the treatment of ALS (Miller *et al.* 2007). However, riluzole showed only a little survival benefit (Miller *et al.* 2007, Zoccolella *et al.* 2007) and patient care has to be supported by palliative therapies throughout the entire course of the pathology. In summary, given this evidence of general failure, from the basic research to the therapeutic approach, a great effort is required in order to change the fate

of this still incurable and fatal disease. Multidisciplinary studies, both on patients and animal models as well as at the cellular level with the use of tissue or cell cultures should be a challenge to obtain more knowledge about the pathogenic mechanisms of the disorder and to devise effective therapies.

1.2 Epidemiology

A wide rate variability in the incidence of ALS was reported since epidemiological studies were based on variable sizes of the study populations and on the use of different diagnostic criteria and methods of case ascertainment (Swash 2000). More homogeneous results have been recently reported from population-based registries, when similar diagnostic criteria and case ascertainment methods were adopted, indicating the incidence of ALS from 1.7 to 2.5 per 100,000 population per year in industrialized European countries (Beghi *et al.* 2007, Logroscino *et al.* 2005). A great variety of disease variants and ALS-like syndromes has been reported over the years leading to up to 10% of misdiagnosed ALS patients. The absence of biological markers of the disease and the complexity of the clinical features concur to render ALS a member of a group of heterogeneous disorders, some of which have common clinical features while others show different clinical and pathological progression of the disease (Beghi *et al.* 2007). Increased age of onset, low forced vital capacity, rapid symptom progression and bulbar site of onset have all been indicated as adverse prognostic factors. However, the rate of disease progression varies greatly among patients. Clinical observations suggest an individual rate of disease progression in most patients. A direct relationship between rate of progression, as determined by isometric myometry (Brooks 1991), and patient survival was shown in many patients enrolled in therapeutic trials (Armon *et al.* 2000, Smith *et al.* 1993).

ALS has an extremely low incidence under age 30 and is almost absent under 20 years. The risk increases highly between 50 and 60 years of age with a clear increase with age until the seventh decade (Beghi *et al.* 2006). However, there is still insufficient evidence to conclude that ALS is an age-related or an aging-related disease. The difficulty in defining ALS in the elderly, due to misdiagnosis with other clinical conditions as well as the presence of numerous comorbidities affecting muscle strength and motion, makes this question even more difficult to answer. The only study (performed in Rochester, Minnesota, US; Yoshida *et al.* 1986) which showed an exponential increase in the incidence of the disease was performed in a small sample of patients. A more recent study (performed on a larger sample) showed a peak in the 60- to 69-year age group, consistent with several other populations (Sorenson et al. 2002).

Males are usually affected more than females (1.6:1), although most recent publications report a significant decrease in the male/female ratio in the 1990s (incidence of ALS in Europe shown in Fig. 1.2).

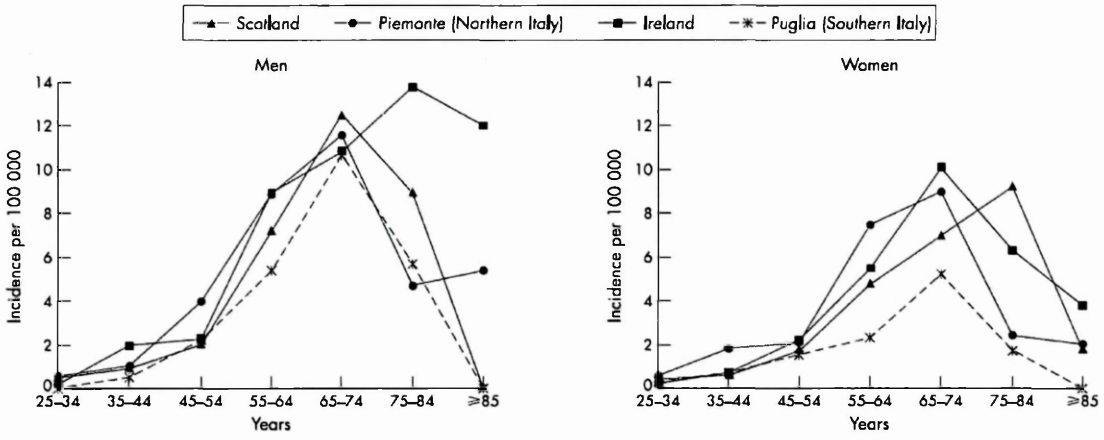


Fig. 1.2 Age specific incidence of ALS for males and females in four European population-based registries (taken from Logroscino *et al.* 2008).

A host of environmental factors have been investigated with alternating fortunes as potential risk factors for ALS (Armon 2001, Mitchell 2000). These include conjugal ALS (i.e., the disease has occasionally been reported in husband and wife), correlation

with antecedent poliomyelitis or concurrent neoplasms (adenocarcinoma, lymphoma, adenoma), exposure to (heavy) metals (including lead, mercury and selenium), to solvents or to electrical or electromagnetic fields, mechanical trauma, heavy physical activity, and living in rural areas or using chemical substances in agriculture. Interestingly, cigarette smoking was the only risk factor supported by fairly good epidemiological evidence (Armon 2003). This finding indicates that the increasing incidence of ALS in women can be related to changing in the contemporary lifestyle which includes exposure to risk factors previously limited to males.

Recent observations of ALS cases in professional soccer players brought into the spotlight the debate whether it could be considered an occupational disease and what are the pathological reasons for its increased incidence in this specific group of athletes. A study on 24,000 Italian professional soccer players who played between 1960 and 1997, found out a tenfold increase in ALS risk since eight soccer players died from the pathology, while the number of cases expected was 0.61 (Belli & Vanacore 2005). This observation was confirmed by recent findings in a cohort of 7325 professional football players (Chio *et al.* 2005) revealing an overall standardized morbidity ratio of 6.5. The position played and the duration of the practice was significantly correlated with the disease risk, with midfielders showing the highest incidence. In addition, the risk was highest in individuals playing 5 years or longer (15.2). A plethora of possible risk factors associated with the participation in competitive sports has been suggested from the literature over the last years. The Body Mass Index and the increased muscularity of the athletes, exposure to herbicides or fertilizers used on playing fields, the frequent occurrence of microtrauma, exposure to dietary supplements or drugs used to enhance sporting performances, have all been proposed as possible events involved in the abnormal incidence of this pathology among the professional soccer players (Chio *et al.* 2005).

Oxidative stress is also associated with strenuous physical activity, and reactive oxygen species are involved in neurodegeneration. In fact, there is indirect evidence of an increased production of reactive oxygen species (ROS) when combining strenuous exercise with other factors (dietary habits, drugs, ischemia followed by reperfusion) (McArdle *et al.* 2001). Oxidative stress can potentially induce detrimental changes in crucial metabolic functions including nucleic acid damage (Cleveland & Rothstein 2001, Julien 2001). However, despite these apparently striking findings no conclusive results have been obtained by individual studies on a single risk factor in the pathogenesis of ALS. A multidisciplinary study seems to be the only practical approach to providing biological support for the epidemiologic evidence and to identify sport-related abnormalities possibly linked to ALS.

Increased risk of ALS was present in Western Pacific areas, especially between the Chamorros, the indigenous population of Guam and other Mariana Islands. The recognition of ALS cases among different ethnic groups with similar socio-cultural habits enforced the hypothesis of the influence of environmental factors, including elements in the soil and water, nutrition and exposure to migratory birds. The role of methylaminoalanine in these foci is of particular interest. Research suggests a cyanobacterial origin for methylaminoalanine in cycad seeds with resultant biomagnification in the food chain (Ince & Codd 2005).

Approximately 5–10% of ALS cases are familial usually showing an autosomal-dominant pattern of inheritance. Autosomal-recessive forms have also been described, particularly from highly consanguineous populations in north Africa (Figlewicz & Orrell 2003). Mutations in the copper/zinc superoxide dismutase (SOD1) gene on chromosome 21 accounts for 10% to 20% of autosomal dominant patients. A number of other genes that cause familial amyotrophic lateral sclerosis (fALS) has also been

discovered and are discussed in detail in Chapter 1.5. Other linkages have also been reported in people with fALS but the genetic lesion in most of these affected individuals remains unknown.

1.3 Clinical features and diagnosis

Although it is now considered a multisystem disease with an important extra motor component, ALS remains a typical motor disorder (Oey *et al.* 2002, Isaacs *et al.* 2007). The clinical features of amyotrophic lateral sclerosis are strictly dependent on the progressive loss of both glutamatergic (motor cortex) and cholinergic (brain stem and anterior horns of the spinal cord) motor neurons. In general, the disease course is characterized by progressive neurodegeneration, muscle weakness with denervation and paralysis (main CNS regions and muscles affected by the pathology in Fig. 1.3),

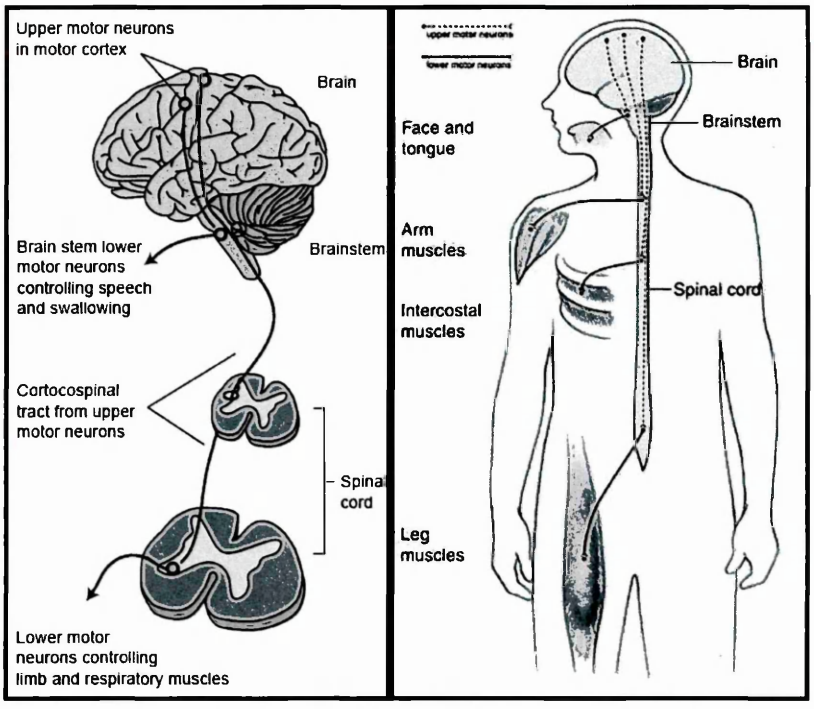


Fig. 1.3 Main regions of the CNS and muscular system affected by ALS (modified from Goodall & Morrison, 2006).

culminating in the patient's death within 3 to 5 years from symptom onset, primarily due to respiratory failure (Mitchell & Borasio 2007).

Although there is a formal separation between sporadic (sALS) and familial groups, both forms show similar clinical and pathological features and seem to share common pathophysiological mechanisms (Ince *et al.* 1998b). The onset of the clinical phase is revealed by functional changes in ALS patients and possible abnormalities in isokinetic muscle tests at a time when isometric strength is not yet reduced in these patients (de Carvalho *et al.* 2003). At the symptomatic phase ALS patients show changes in isokinetic strength as well as early changes in isometric strength. During this phase, pathology is usually reported to be more severe in the anatomical areas where ALS had initially started but progressively increases even in regions to which the symptoms had spread (Ince *et al.* 1998a). Patients presenting bulbar onset show slurring of speech (dysarthria), difficulty swallowing (dysphagia), or both. Bulbar involvement is associated with upper and lower facial weakness and poverty of palatal movement with wasting, weakness, and fasciculation of the tongue. When upper motor neurons are mainly involved, patients present emotional lability (also known as pathological laughing or crying), brisk jaw jerk, and dysarthria. Cervical-onset patients present upper-limb symptoms, either bilateral or unilateral. Proximal weakness with difficulty in shoulder abduction (e.g., hair washing, combing, etc) and profuse arm fasciculation and distal weakness with impairment of activities requiring pincer movements are shown. Lumbar onset implies degeneration of the anterior-horn cells of the lumbar enlargement and is associated with lower motor neuron symptoms and signs in the legs, such as a tendency to trip (foot drop) or difficulty on stairs.

Although involvement of other systems is not considered in ALS clinical observations, recent studies have revealed the concomitant presence of unrelated symptoms, including

sensory dysfunction, autonomic nervous system abnormalities, mood alterations and cognitive abnormalities (Daube 2000, Mulder *et al.* 1983).

The progressive atrophy of respiratory muscles results in respiratory failure, the most common cause of death in ALS patients. Measurements of forced vital capacity are useful to identify early respiratory problems (Borasio & Voltz 1997). Dyspnoea at rest is the earlier hallmark of respiratory failure. As the respiratory difficulty increases, attacks of sleep apnoea do not allow patients to sleep supine. During the development of the disease, when respiratory forced activity is completely lost, patients need assisted ventilation (Gelanis 2001).

Diagnosis of ALS is usually based on clinical evaluations supported by electrophysiological assessments. As a consequence of the lack of a specific test or biological markers, up to 10% of patients initially diagnosed with ALS might be affected by different disorders (Bradley *et al.* 2004).

Progressive spinal muscular atrophy, cervical radiculomyelopathy, thoracolumbar-sacral disc disease, multifocal motor neuropathy, chronic inflammatory demyelinating neuropathy, adult-onset spinal muscular atrophy, myasthenia gravis, spinobulbar muscular atrophy (Kennedy's disease), multiple sclerosis, cerebrovascular disease, and multiple system atrophy have been misdiagnosed as ALS (Traynor *et al.* 2000, Visser *et al.* 2002). The factors that guide clinicians to the correct diagnosis include the lack of progression, the development of atypical symptoms, and the results of additional investigations. In fact, for the correct identification of ALS, signs of upper and lower motor neuron involvement and a progressive spread of neurological signs from the bulbar or spinal region of onset towards other areas are required.

Assessment of disease progression is also difficult. Several different functional scales, new neurophysiological tests, and imaging techniques have been developed but none

of these has sufficient diagnostic or prognostic certainty (Mitsumoto *et al.* 2007, Winhammar *et al.* 2005). To date, the most useful clinical measures are vital capacity (Czaplinski *et al.* 2006) and the revised ALS functional rating scale (Cedarbaum *et al.* 1999, Kaufmann *et al.* 2005). Functional rating scales are excellent primary outcome measures since the complexity of ALS clinical features requires a pragmatic classification containing precise diagnostic criteria. Such determination was first devised in El Escorial (Madrid) in 1990 (Brooks 1994). The "El Escorial" scale is based on different parameters, clinical features that allow classification of the probability of a patient having ALS, according to the degree of clinical certainty in relation to other pathologies, such as multiple sclerosis, cervical myelopathy and other similar neuromuscular disorders (Brooks 1994). The "El Escorial" scale includes four degrees of certainty for the diagnosis of ALS. They can range from "clinically definite" to "clinically suspected". In 1994 neuropathological analyses were included in the "El Escorial" diagnostic criteria (World Federation of Neurology Research Group on Neuromuscular Disease Subcommittee on Motor Neuron Disease). The list of neuropathological features supporting the diagnosis included selective atrophy of the motor cortex, greyness and atrophy of anterior spinal nerve roots compared with normal roots, grains of the lateral columns of the spinal cord and atrophy of skeletal muscles. Features that rule out the diagnosis of ALS or suggest the presence of an additional disease were considered to be plaques of multiple sclerosis or a focal cause of myelopathy.

Because of the inability to confirm a diagnosis of ALS exclusively by gross examination of the brain and spinal cord tissues, light and electron microscopy analyses are needed. Although it represents a valid criterion to diagnose ALS, this approach cannot evaluate the progression of symptoms. To determine a more efficient protocol to

score the progression of symptoms, "El Escorial" criteria have subsequently been revised and to date the ALS Functional Rating Scale (ALS FRS) is the most widely used in clinical trials. Although intended as an aid to research and slightly more restrictive than the burden of proof usually applied in clinical practice, these criteria do provide a structured approach to assessment of people suspected of having amyotrophic lateral sclerosis, which can enhance objectivity in clinical practice and facilitates clinical studies. The ALS FRS is a ten-item scale with five different scores for each item. The total score is obtained by the sum of scores for each single item and increases proportionally to the symptomatological worsening. The evaluation of three bulbar functions (speech, salivation and swallowing), one respiratory function (breathing), three upper-extremity (handwriting, cutting food and dressing), one truncal function (turning in bed) and two lower-extremity (walking and climbing stairs) are included in ALS FRS (ALS CNTF Treatment Study group-no authors listed).

The aim of this scale is: I) to provide a unique, simple, reproducible, sensitive and specific protocol to test the possible efficiency of drugs during clinical trials; II) to better characterize and separate the evolution of symptoms among the different forms of ALS; III) to define the different stages of disease for a population of patients who might have all types of onset.

1.4 Histo- and cyto-pathological hallmarks

In the study of neurodegenerative diseases, knowledge of the descriptive neuropathology is essential to draw out the clinical pattern of the disease, to differentiate among distinct pathologies sharing similar symptoms and to understand whether neuropathological observations found in animal models are similar to the human condition. From a pathological point of view ALS is mainly characterised by degeneration and loss of motor neurons associated with gliosis. Intracellular inclusions

were found in degenerating neurons and glia. Abnormalities in mitochondria and axons, and alterations in neurofilament composition were revealed in ALS patient specimens and in transgenic mice with a mutation in SOD1. The main neuropathological alterations in both upper glutamatergic as well as lower cholinergic motor neurons are described in detail below.

Upper motor neurons and cortico-spinal tract

In the cerebral cortex a marked decrease in large pyramidal motor neurons has been predominantly observed in the motor area. However, neuronal abnormalities are not confined to the motor cortex; in fact, a lesser degree of damage was also reported in the premotor areas, sensory cortex and temporal cortex. Quantitative histological studies of cortical motor neurons revealed changes in size with shortened and fragmented dendrites. Accumulation of neurofilaments and ubiquitinated proteins are common before motor neuron death (Kiernan & Hudson 1991). Demyelination, due to axonal degeneration of descending large myelinated fibers of cortical motor neurons, is a common feature in the cortico-spinal tract of ALS patients. Moreover, cortico-spinal fibres showed a marked axonal swelling and spheroids containing packed neurofilamentous material and other cellular debris are found (Chou 1992).

Lower motor neurons

A marked loss of spinal motor neurons showing a patchy and focal pattern was found in the anterior horn of ALS patients (Tsang *et al.* 2000). Quantitative histological studies showed that large motor neurons are selectively involved. In these cells neuronal shrinkage or atrophy precedes neuronal death and also involves alterations to axons and dendrites. Loss of Nissl substance (chromatolysis), vacuolization and lipofuscin deposits are sometimes detectable in the remaining anterior horn motor neurons

(Kiernan & Hudson 1991). Since spinal motor neurons represent the best-studied and characterized group of cells affected by the pathology, a detailed description of ultrastructural alterations found in these neurons is reported below.

Neurofilament alterations

Cytoskeletal components direct several processes of eukaryotic cells, including mitosis, cell motility, endocytosis, and the maintenance of cellular shape. Moreover, in neurons, cytoskeletal organisation is fundamental to connect the cellular body to the periphery, axons and dendrites, and to regulate the transport of a large number of molecules by an extensive network of microtubules and filaments (Bloom & Goldstein 1998). Neurofilaments are the most abundant filamentous component in neurons. They are divided into three different classes, light neurofilaments (NF-L) medium neurofilaments (NF-M) and heavy neurofilaments (NF-H) depending on their molecular size (Al-Chalabi & Miller 2003). In healthy motor neurons neurofilaments are expressed in the perikaryon and are then transported toward the periphery in a not-phosphorylated form. Neurofilament phosphorylation is a crucial event for the assembling of single neurofilaments taking place when they reach the target cellular localization. This mechanism is essential for the extension of axons and to maintain the integrity of neuronal morphology (Shea *et al.* 2003). In recent years a growing body of evidence suggests that various neurodegenerative diseases share a similar process in which neurofilaments are phosphorylated and accumulated in the cell body of dying neurons (Taylor *et al.* 2002, Zhao *et al.* 2001). A common pathological feature, observed in autoptic samples of ALS patients, is the presence of a marked accumulation of intermediate filaments in the perikaryon of motor neurons and in their axons (Gambetti *et al.* 1983, Hirano *et al.* 1984). Immunostaining experiments with monoclonal antibodies directed against the three different forms of neurofilaments showed a 5 to 10

fold increased immunopositivity in motor neurons from ALS patients compared to those from control patients (Rouleau *et al.* 1996). Abnormal neurofilaments accumulation in the perikaryon occurs in two distinct patterns: homogeneously diffuse or focally accumulated in various shapes. Neurofilaments are not the unique category of cytoskeletal components present in ALS inclusion bodies. Peripherin is another protein detected in the majority of inclusions in motor neurons of ALS patients (Corbo & Hays 1992, Migheli *et al.* 1993). Peripherin is normally expressed in peripheral sensory neurons but is almost absent in the motor neurons (Escurat *et al.* 1990, Troy *et al.* 1990). However, peripherin gene expression can be up regulated by nerve injury and inflammatory cytokines (Wong & Oblinger 1990).

Intracellular inclusions

Several types of inclusion bodies that were found in the perikarya of anterior horn motor neurons are immunoreactive to ubiquitin (Leigh *et al.* 1989). Ubiquitin is a 76-amino acid-protein that is involved in an ATP-dependent nonlysosomal proteolysis of abnormal or short-lived proteins (Leigh *et al.* 1991). Although ubiquitin inclusions in lower motor neuron are unique to ALS tissues, ubiquitin-immunoreactive intraneuronal inclusions were often observed in the upper motor neurons (Morris *et al.* 2001) and are not specific to this disease (Wilson *et al.* 2001). By immunoelectron microscopy observation, it was shown that these ubiquitin-reactive inclusions are composed of small 10-15 Å linear fragments whose exact identity remains undiscovered (Okamoto *et al.* 1991a). Lewy-body-like hyalin inclusions were originally described as a characteristic feature of posterior columns and spinocerebellar tracts in ALS patients, but furthermore they have also been found in the soma and in the proximal axons of anterior horn motor neurons (Murakami 1990). These inclusions measure 7 to 20 μm in diameter and are surrounded by a lighter, slightly basophilic halo and contain a dense eosinophilic core

of granules associated with a patchy shaped aggregate of filaments. These filaments are not immunoreactive to any cytoskeletal components such as neurofilaments, tubulin, microtubule-associated protein 2, or phosphorylated tau protein. These inclusions are similar to those described in the Lewy bodies in Parkinson's disease.

Bunina bodies are hystologically described as small, eosinophilic, irregularly shaped, 2 to 3 μm in diameter and exclusively found in the perikaryon. Ultrastructurally, they are electron dense amorphous structures surrounded by lysosomal vesicles, endoplasmic reticulum fragment, lipofuscin granules and other debris.

An intriguing relationship between bunina bodies and Golgi apparatus fragments has been hypothesised because of the presence in the bunina bodies of marked immunoreactivity for anti-cystatin C antibody, a protein highly expressed in the Golgi apparatus (Okamoto *et al.* 1991b).

Mitochondrial alterations

Abnormalities in mitochondrial morphology and alterations in electron transport chain activities were found both in muscle samples from patients with ALS and in the transgenic SOD1 mouse model of the pathology. When analysed by electron microscopy, muscle mitochondria showed ultrastructural abnormalities including increased size, paracrystalline inclusions and abnormal cristae (Mourelatos *et al.* 1996). Similar observations come from the G37R and G93A mouse models where membrane-bound vacuoles in axons, dendrites and motor neurons appeared to be derived from degenerating mitochondria (Bendotti *et al.* 2001, Kong & Xu 1998, Wong *et al.* 1995). A fine ultrastructural examination of the synapses in ALS patients showed mitochondrial alterations in the anterior horn motor neurons (lumbar spinal cord) not only in degenerated neurons, but, to a lesser extent, in neurons that appear normal. Mitochondrial abnormalities include dense conglomerates of aggregated, dark

mitochondria and presynaptic vesicles, and also bundles of neurofilaments, and a marked increase in presynaptic vesicles. These observations suggest that a substantial synaptic alteration, including mitochondrial changes, occurs in the early stages of anterior horn neuron death process in ALS patients (Swerdlow *et al.* 1998).

Impairment of mitochondrial function was observed in skeletal muscle biopsies from patients with ALS (Borthwick *et al.* 1999, Wiedemann *et al.* 1998), and altered respiration was described in muscle mitochondria of patients with early stage sALS using the skinned fiber technique (Echaniz-Laguna *et al.* 2002). Loss of citrate synthase activity (a mitochondrial marker) as well as decreased activities of respiratory chain complexes I and III, II and III, and IV suggested a loss of mitochondria in spinal cords of patients with ALS (Wiedemann *et al.* 2002). Mitochondrial respiration, electron transport chain, and adenosine triphosphate synthesis were defective in G93A mice at the onset of the disease (Mattiazzi *et al.* 2002). In the same animal model, mitochondrial electron transport chain activities were decreased in the spinal cord ventral horn prior to disease onset and during disease progression (Jung *et al.* 2002). These alterations in mitochondrial function in G93A SOD1 mice appeared to be specific and preferentially targeted to the central nervous system (Kirkinezos *et al.* 2005).

Mitochondrial respiration is the main source of reactive oxygen species (ROS) in the cell, and ROS levels tend to increase when respiration is impaired (Wei *et al.* 1998). Both the involvement of mitochondria and the association of SOD1 with ALS suggest that oxidation of macromolecules could have a role in the pathogenesis of ALS. In patients with sALS, both lipid and protein oxidation are enhanced in the spinal cord motor neurons and glial cells, suggesting that the formation of these products is implicated in motor neuron degeneration (Shibata 2001). Markers of oxidative stress and immune activation were significantly elevated in post-mortem ALS tissue in the CNS (Siciliano *et al.* 2002) and abnormally increased blood levels of ROS and lactate

production may indicate a close relationship between mitochondrial function and oxidative stress in ALS (Simpson *et al.* 2004). These increases in ROS and products of oxidation have been observed both in post-mortem samples and in experimental models for ALS and may result from an altered metabolism of copper and iron ions (Carri *et al.* 2003).

Axonal alterations

As in other neurodegenerative diseases, axonal swelling represents a common event detectable in ALS. In ALS patients the axons are swollen and typically show well-defined structures that are identified as spheroids (Carpenter 1968). These structures are eosinophilic bodies and are usually greater than 20 μm in diameter. They contain packed 10-nm-thick neurofilaments. Like intraneuronal neurofilament accumulations, spheroids are phosphorylated. However, spheroids have not been found exclusively in ALS tissues and many other neurological diseases are characterized by the presence of phosphorylated neurofilaments and neurofilamentous conglomerates (Chou 1992, Manetto *et al.* 1988).

Protein aggregates

As reported for other neurodegenerative diseases, like Alzheimer's disease, prion disease, Kennedy and Huntington's disease, protein accumulation in motor neurons of ALS patients may be caused by the formation of aggregates due to an alteration in the process of misfolded protein degradation (Ross & Poirier 2004). The mutant SOD1 protein was reported to sequester chaperones that are required for promoting the correct folding of many proteins, whereas ubiquitin-mediated protein degradation might be inhibited by these aggregates. Aggregates intensely immunoreactive for SOD1 are

found in the motor neurons of fALS patients carrying SOD1 mutations, and ubiquitin deposits have been reported in lower motor neurons in both sALS and fALS patients. The evidence of a large amount of ubiquitin positive aggregates in motor neurons of ALS patients strengthens the hypotheses that a failure in the ubiquitin-proteasome system can be responsible for protein accumulation (Migheli *et al.* 1999, Valentine & Hart 2003).

α-Synuclein is a small (14 kDa) protein mainly expressed in the brain and predominantly concentrated in presynaptic nerve terminals. At physiological concentrations α-synuclein is an unfolded protein with no ordered secondary structure but it is well known that it can undergo polymerisation into fibrils associated with the formation of toxic aggregates (Moran *et al.* 2001). α-Synuclein aggregates are associated with several neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, Lewy body dementia, multiple system atrophy and it has been recently associated with ALS (Bennett 2005). Aggregates of α-synuclein have been observed in neuronal spheroids, astrocytes, Schwann cells and in cortico-spinal axon tract fibers and glia in brain and spinal cord of ALS patients (Doherty *et al.* 2004, Mezey *et al.* 1998). In addition an increased expression of α-synuclein has been detected in the anterior horn in the spinal cord of SODG93A transgenic mice, an animal model of ALS (Chung *et al.* 2003).

1.5 Etiology

Despite extensive research, the pathogenic mechanisms of ALS are still unknown and this is probably the main reason that could account for the lack of effective treatments. The process of motor neuron degeneration looks complex and multifactorial and remains poorly understood in terms of a unifying causal hypothesis suggesting, indeed, that it might be a common end-stage phenotype of different causes. As for other

complex diseases, the etiology of ALS is likely to involve both genetic and environmental factors (overview of the main factors involved in ALS etiology in Fig. 1.5.1).

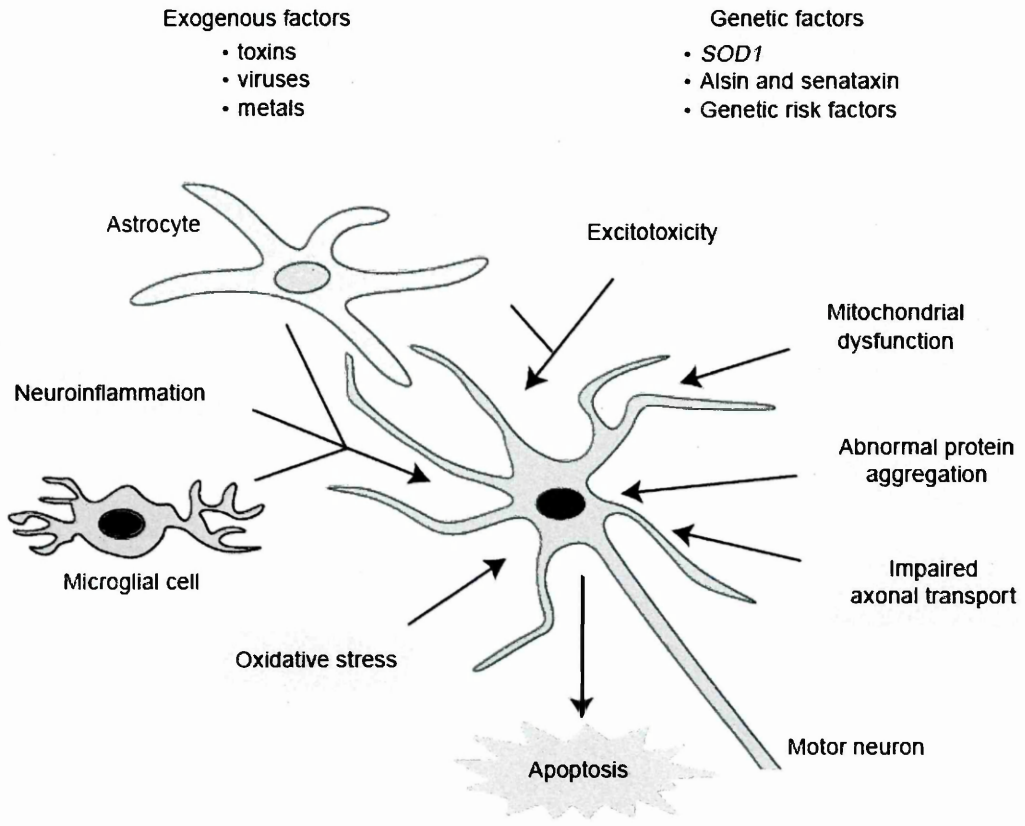


Fig. 1.5.1 Overview of the main mechanisms contributing to ALS pathogenesis (taken from Goodall & Morrison, 2006).

Genetic forms

Between 5 and 10% of ALS cases are familial, most often compatible with autosomal dominant inheritance. The disease progression and features are clinically and pathologically very similar between the familial and the sporadic cases, suggesting that they share common pathogenic mechanisms. Many current studies are focused on the detection of genetic factors in ALS, to seek loci and genes responsible for the familial

forms and to identify genetic polymorphisms as risk factors in the more common sALS. A key discovery in the field was the identification of mutations in the Cu/Zn superoxide dismutase 1 (SOD1) gene as the cause of approximately 20% of fALS and therefore 2% of all cases (Rosen *et al.* 1993). SOD1 is part of the cellular defence against oxidative stress; it catalyses the conversion of superoxide anions into hydrogen peroxide, which is then further metabolised. More than a hundred mutations in SOD1, distributed throughout the gene, have been found in fALS patients (Borchelt *et al.* 1994). It is still not known why the mutant form of this abundant and ubiquitously expressed enzyme should be particularly toxic to motor neurons and causes ALS. Extensive study has provided strong evidence for a toxic gain of function rather than a loss of enzymatic function of the mutant enzyme. SOD1 enzymatic activity varies greatly depending on which mutation is present and does not correlate with disease severity, with some mutant forms maintaining full activity (Borchelt *et al.* 1994). Knockout mice do not develop overt ALS (Reaume *et al.* 1996), while transgenic mice overexpressing mutant forms of the SOD1 protein (mSOD1) develop an adult-onset progressive motor neuropathy phenotype (Gurney *et al.* 1994).

Double-transgenic mice containing both mutant and wild-type SOD1were produced and showed a phenomenon whereby human wild type SOD1 exacerbates ALS-like disease in different transgenic mice. This phenomenon is accompanied by a conversion of the human wild type SOD1 from a soluble form to aggregates of dimers and multimers (Deng *et al.*, 2006). Analysis of transgenic mice expressing familial amyotrophic lateral sclerosis (ALS)-linked mutations in the enzyme superoxide dismutase (SOD1) have shown that motor neuron death arises from a mutant-mediated toxic property or properties (Bruijn *et al.*, 1998). Transgenic rats overexpressing various human mSOD1 genes have also now been developed, showing features similar to the human disease (Howland *et al.* 2002, Nagai *et al.* 2001). There are many theories as to why mSOD1 is

toxic, including enhanced oxidative stress from aberrant free radical production, and protein misfolding leading to abnormal aggregation. There are also data indicating a non-cell-autonomous toxic pattern of mSOD1. In fact, motor neuron-specific expression of mSOD1 does not produce ALS in mice and neurodegeneration is delayed or eliminated when motor neurons expressing mSOD1 are surrounded by wild-type cells (Clement *et al.* 2003, Pramatarova *et al.* 2001).

Genetic linkage studies in pedigrees in which motor neuron disorder phenotypes are segregated have identified other disease-causing loci and genes. For example, mutations in alsin cause autosomal recessive juvenile-onset forms of ALS and the upper motor neuron variant primary lateral sclerosis, and mutations in senataxin cause a slowly progressive autosomal dominant disorder. Alsin encodes a protein with three putative guanine-exchange factor domains that may activate small GTPases and have a role in signal transduction (Yang *et al.* 2001). Several groups have now generated alsin knock-out mouse models, but only mild neurological changes have been reported in these animals to date (Cai *et al.* 2005, Hadano *et al.* 2006). Senataxin contains a DNA/RNA helicase domain, which may suggest mutations cause a defect in RNA processing (Chen *et al.* 2004).

Genetic association studies in ALS helped to identify the main pathways affected by the pathology. Genetic polymorphisms associated with ALS include deletions or insertions in the neurofilament heavy chain gene, deletions in the promoter of vascular endothelial growth factor (VEGF), mitochondrial DNA deletions, a polymorphism in the haemochromatosis gene HFE and, recently, mutations in the angiogenin gene (Dhaliwal & Grewal 2000, Figlewicz *et al.* 1994, Goodall *et al.* 2005, Greenway *et al.* 2006, Lambrechts *et al.* 2003).

Pathogenic hypotheses

Different pathogenic mechanisms have been proposed to contribute to ALS onset. Current works focuse largely on excitotoxicity and mitochondrial alterations with consequent oxidant stress. The excitotoxic hypothesis has led to the identification of riluzole, a glutamate-release inhibitor, as the first licensed disease-modifying treatment for amyotrophic lateral sclerosis (reviewed by (Miller et al. 2007). Attempts to develop antioxidant strategies for the disorder have, by contrast, been disappointing (Orrell *et al.* 2005). Viral hypotheses drawing from the role of poliovirus in poliomyelitis have been pursued extensively without positive evidence emerging. Other pathogenic hypotheses have been related to neurofilament alterations, protein aggregation and an immunoinflammatory process.

Excitotoxicity

Glutamate-induced excitotoxicity is the best-characterized factor in the ALS pathogenesis. Glutamate is the main excitatory neurotransmitter in the mammalian CNS, acting through both ligand-gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors. The ionotropic glutamate receptors are multimeric assemblies of four or five subunits, named AMPA, NMDA or Kainate receptors, dependently on their pharmacology structural similarities. The subunits share a common basic structure with an extracellular N-terminus and intracellular C-terminus. NMDA receptors (NMDAR) are composed of assemblies of NR1 and NR2 subunits, the last being one of four separate gene products (NR2A-D).Expression of both subunits is required to form functional channels. In addition to glutamate the NMDAR requires the co-agonist glycine to bind to NR1 subunit to activate the receptor. At resting membrane potentials, NMDAR are inactivate because of a voltage-dependent block by magnesium ions. Sustained activation of AMPAR depolarises the post-

synaptic membrane, releasing the channel inhibition and thus allowing NMDAR activation and the following influx of calcium and other ions.

AMPAR are composed of subunits GluR1-4, products from separate genes. The ligand-binding domain is made up from N-terminal regions while the C-terminus contains binding sites for proteins involved in AMPAR trafficking and targeting. Native AMPAR channels are impermeable to calcium, a function controlled by the GluR2 subunit. The calcium permeability of the GluR2 subunit is determined by the post-transcriptional editing of the GluR2 mRNA at the so called Q/R editing site. Glutamine (Q) - containing GluR2 is calcium permeable while arginine (R) - containing GluR2 is not. Different editing forms of the subunits as well as different subunit assembly of the receptor confer to AMPAR peculiar properties within the different cells.

Kainate receptors (Abhyankar *et al.*) are built from multimeric assemblies of GluR5-7 and KA1-2 subunits. They undergo both splice variation and RNA editing, conferring to the receptor different pharmacological and functional properties. Little is known about the specific physiological and/or pathological role of this receptor since kainate is also active on AMPAR and exerts many of its effect through the activation of both KAR and AMPAR. For this reason the two receptors are usually considered as common AMPA/KA receptors in neurodegenerative studies.

Excitotoxicity is the process by which amino acid neurotransmitters such as glutamate become toxic when present at supraphysiological concentrations inducing overstimulation of postsynaptic glutamate receptors (representation of the main cellular alterations induced by excitotoxicity is shown in Fig. 1.5.2). This overstimulation can generate an excessive influx of Ca^{2+} and Na^{+} into the neurons and the following activation of damaging pathways ultimately leading to cell death (Choi 1987). Both glial and neuronal glutamate transporters play a pivotal role in avoiding excitotoxicity by removing the excess of glutamate released into the synaptic cleft from presynaptic

neurons and, consequently, by preventing the overstimulation of postsynaptic glutamate receptors. Evidence of alterations in glutamate homeostasis both in patients and in animal models have accumulated over the years. Increased levels of glutamate have been found in the CSF of 40% of sALS patients (Spreux-Varoquaux *et al.* 2002). Abnormal glutamate metabolism and selective loss of the glial glutamate transporter (EAAT2) in the anterior horn of affected spinal cord regions in ALS patients were reported (Rothstein *et al.* 1995).

In addition, a significant decrease in glutamate reuptake from cerebral and spinal synaptosomes from autoptic samples has been measured (Rothstein *et al.* 1992).

Glutamate overstimulation produces its neurotoxic effect acting through the N-methyl-D-aspartate (NMDA) receptor and the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate ionotropic receptors. However, while the NMDA receptor seems to mediate most acute neuronal injury, AMPA/kainate receptors (AMPARs) seem to be associated with detrimental effects produced following a slower and prolonged overstimulation.

This latter mechanism appears to be more related to chronic processes, such as neurodegenerative diseases and ALS (Weiss & Choi 1991).

AMPARs are cation-conducting complexes; the receptors are composed of four subunits (GluR1-4) and are always constituted as an heteromeric protein. The alternative assembly of the different subunits produces important differences in the electrophysiological properties of AMPAR (Jonas & Burnashev 1995). A possible mechanism of excitotoxicity due to post-transcriptional editing of the calcium impermeable subunit (GluR2) of AMPAR has been postulated (Takuma *et al.* 1999). Ca^{2+} permeability is largely determined by the GluR2 subunit, receptors incorporating an edited GluR2 subunit show a limited Ca^{2+} permeability in comparison with GluR2-lacking channels or containing the unedited form of GluR2.

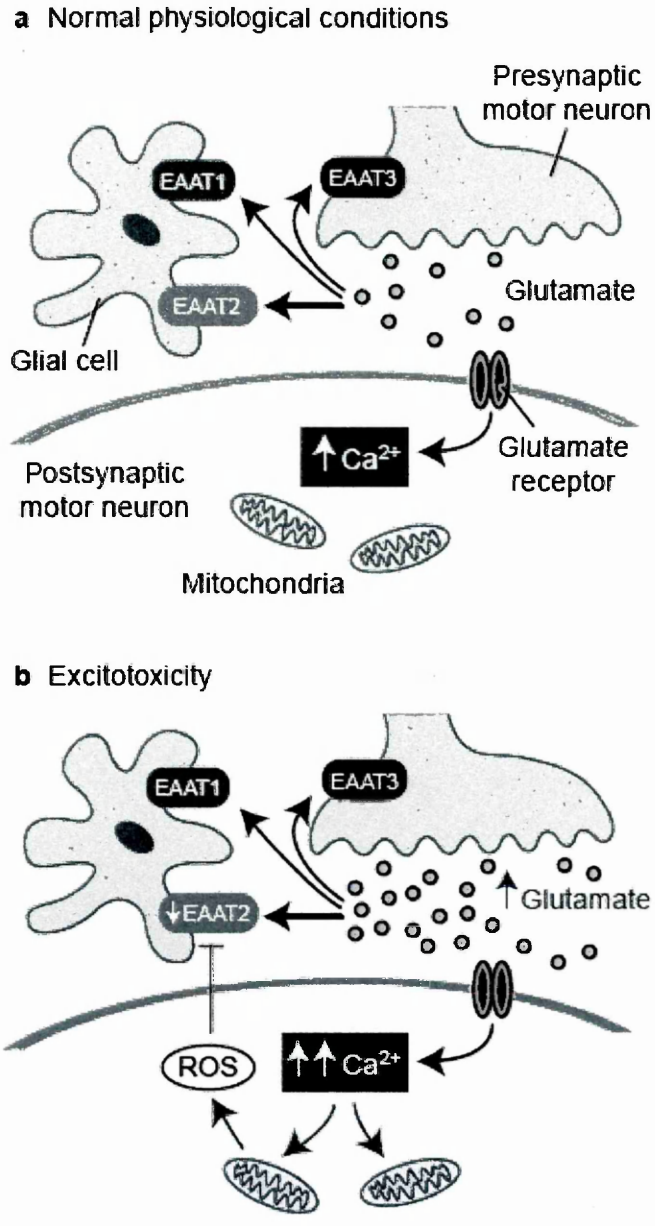


Fig. 1.5.2 Glutamatergic transmission in the CNS under physiological (a) or pathological (b) conditions (taken from Goodall & Morrison, 2006)

This effect is related to the presence of an arginine in its pore-forming segment in GluR2, in a position occupied by a glutamine in the other AMPAR subunits (Burnashev *et al.* 1992, Hume *et al.* 1991). This critical arginine residue is formed at the pre-mRNA stage by RNA editing (Sommer *et al.* 1991). The relative Ca^{2+} permeability of native

AMPARs in neurons is inversely correlated with the rate of edited GluR2 and the divergence in relative Ca^{2+} permeability of AMPAR between different neuronal cell types could be an important constituent of selective vulnerability (Pellegrini-Giampietro *et al.* 1997). It has been widely demonstrated that different neuronal cell types can diverge in GluR2 expression, in the rate of GluR2 editing and in the desensitisation properties of their AMPAR (Raman *et al.* 1994) and such differences may be related to the selective vulnerability of motor neurons that occurs in ALS.

The prolonged elevation of motor neuron intracellular calcium (which seems to represent the irreversible step of the degenerative pathway, Lukas & Jones 1994) starts a complex cascade of correlated intracellular events such as the activation of numerous enzymes (nucleases, cytosolic proteases and kinases, lipases etc.; Favaron *et al.* 1990, Mills & Kater 1990, Orrenius *et al.* 1989) and calcium-dependent protein interactions, which can compromise cellular homeostasis.

Furthermore, exposure of mitochondria to high concentrations of intracellular free calcium levels leads to increased free radical production and causes impairment of neuronal energy metabolism, which may sensitise neurons to excitotoxic cell death (Arundine & Tymianski 2003, Dykens 1994).

Mitochondrial alterations and oxidative stress

Mitochondrial main activities involve providing energy through oxidative phosphorylation, maintaining calcium homeostasis, producing free radicals and regulating cell death pathways. Mitochondrial respiration is the main source of reactive oxygen species (ROS) in the cell, and ROS levels tend to increase when respiration is impaired (Wei et al. 1998). Morphological and ultrastructural abnormalities of mitochondria in SALS were initially observed in autopsies. Subsarcolemmal aggregates of abnormal mitochondria were found in skeletal muscle and in intramuscular nerves

(Afifi *et al.* 1966, Atsumi 1981). Mitochondrial morphological abnormalities were also detected in proximal axons (Hirano et al. 1984) and in the anterior horns of the spinal cord in sALS patients (Atsumi 1981, Sasaki & Iwata 1996). Increased mitochondrial volume and elevated calcium levels within the mitochondria were found in muscle biopsies of ALS patients (Siklos *et al.* 1996). Deficits in the activities of mitochondrial respiratory chain complex I (Wiedemann et al. 1998) and complex IV (Vielhaber *et al.* 2000) have been identified in the skeletal muscle and in the spinal cord of sALS patients (Borthwick et al. 1999, Wiedemann et al. 2002). The observation of increased resting cytosolic calcium and reduced response to uncouplers of oxidative phosphorylation in lymphocytes from sALS patients accounts also for impairment of mitochondrial functions (Curti *et al.* 1996).

The evidence of mitochondrial alterations together with the involvement of mutations of SOD1 in ALS cases support the hypothesis that oxidation of macromolecules could have a role in the pathogenesis of ALS. In patients with sALS both lipid and protein oxidation are enhanced in the spinal cord motor neurons and glial cells, suggesting that the formation of these products is implicated in motor neuron degeneration (Shibata *et al.* 2001). Markers of oxidative stress and immune activation were significantly elevated in post-mortem tissue in the CNS (Simpson et al. 2004), and abnormally increased blood levels of ROS and lactate production (Siciliano *et al.* 2002) were found in ALS patients. Increase in ROS and products of oxidation have been observed also in experimental models for ALS and may result from an altered metabolism of copper and ferrous/ferric ions (Carri *et al.* 2003). mSOD1 transgenic mice, indeed, show elevated levels of protein and lipid oxidation at both pre- and post-symptomatic stages (Gajewski *et al.* 2003).

Cell culture studies have been conducted to determine the functional significance of mitochondrial (mt) DNA changes in ALS using cytoplasm hybrid (cybrid) cell lines.

Patient's and control platelets (containing no nucleus) were fused with neuroblastoma cells (with depleted mitochondria) to create cybrid cells differing only in their mtDNA. The ALS cybrid cells had several defects including altered mitochondrial ultrastructure, decreased complex I activity and decreased calcium storage by the mitochondria (Paradies *et al.* 2000).

Vacuolated mitochondria are a striking and early feature of disease in some strains of mSOD1 mice, including the most commonly used G93A model. The degree of vacuolation correlates with the decline in muscle strength (Crapo *et al.* 1992). Studies using in vitro cellular models have also shown that mSOD1 disrupts mitochondrial morphology and function (Fridovich 1997, Kira *et al.* 2002).

Correlations between oxidative stress and other proposed disease mechanisms such as excitotoxicity and axonal transport defects have been proposed. Excitotoxicity-dependent increased intracellular calcium may lead to increased nitric oxide formation. Peroxynitrite, generated by the reaction of superoxide anions and nitric oxide, can subsequently be the trigger of oxidative damage (Rao & Weiss 2004). Nitration may also target neurofilament proteins, disrupting their phosphorylation and affecting axonal transport (Torreilles *et al.* 1999).

Neurofilament alterations

Both in sporadic and in familial forms, neurofilament alterations represent a pathological hallmark of ALS (Julien & Beaulieu 2000). Neurofilament proteins (neuron-specific intermediate filaments) are the most abundant structural protein in mature motor neurons, and aggregates of neurofilament proteins in the cell body and proximal axons of motor neurons are commonly seen in ALS. Neurofilament heavy chain gene deletions were found in 1% of sALS cases (Al-Chalabi *et al.* 1999, Figlewicz *et al.* 1994) supporting a direct involvement of neurofilaments in the

pathogenesis of ALS. However, how this aberrant expression of neurofilament can lead to a selective damage for motor neurons is still unclear.

Experiments in transgenic mice revealed a key role for neurofilaments in normal motor neuron function. Mice overexpressing human wild-type or mutant neurofilament protein develop motor neuropathies and undergo massive motor neuron cell death (Cote *et al.* 1993, Lee *et al.* 1994, Xu *et al.* 1993). Peripherin and α-internexin are two intermediate-filament proteins that co-localise with neurofilaments and form part of the axonal inclusion bodies in ALS. Overexpression of peripherin or α-internexin in transgenic mice causes motor neuron degeneration (Beaulieu *et al.* 1999, Ching *et al.* 1999). Peripherin is encoded by a single gene and has splice variants of 56, 58 and 61 kDa. Peripherin 61 is toxic to primary motor neuron cultures, even at low levels, and has been detected in the spinal cord of sALS patients (Robertson *et al.* 2003). When considering the role played by neurofilaments in ALS pathology it is important to note that the formation of intraneuronal aggregates which are highly enriched in phosphorylated neurofilaments, is a peculiarity of motor neurons in ALS patients. This increase in highly phosphorylated neurofilaments could also suggest that the failure in the metabolism of neurofilaments may be the first step in a series of processes leading to neurofilament accumulation, axonal strangulation and neuronal cell death. However, it has been also suggested that upstream defects, such as, axonal transport deficit, glutamate induced excitotoxicity, SOD1 mutation or inflammatory response, might cause the disorganisation of neurofilaments in the cell body; in which case neurofilament alteration would represent more of an effect than a cause of the disease (Ackerley *et al.* 2000, Asahara *et al.* 1999).

Aberrant protein aggregation

Abnormal protein aggregates are recurrent etio-pathological signs of ALS and include Bunina bodies, ubiquitinated inclusions and neurofilament inclusions. As reported for other neurodegenerative diseases, like Alzheimer's disease, prion disease, Kennedy and Huntington's disease, protein accumulation in motor neurons of ALS patients may be caused by the formation of aggregates due to an alteration of the process of misfolded protein degradation (Ross & Poirier 2004).

SOD1

The mutant SOD1 protein was reported to sequester chaperones that are required for promoting the correct folding of many proteins, whereas ubiquitin-mediated protein degradation might be inhibited by those aggregates. Aggregates intensely immunoreactive for SOD1 are found in the motor neurons of fALS patients carrying SOD1 mutations, and ubiquitin deposits have been found in lower motor neurons in both sALS and fALS patients. The evidence of a large amount of ubiquitin-positive aggregates in motor neurons of ALS patients strengthens the hypothesis that a failure in the ubiquitin-proteasome system can be responsible for protein accumulation (Valentine & Hart 2003, Migheli *et al.* 1999).

TDP-43

The TAR DNA binding protein (TDP-43) is an ubiquitously expressed nuclear protein capable of binding DNA and RNA. TDP-43 is the major protein in ubiquitinated inclusions in neuronal cytoplasm of ALS patients, and it is usually accompanied by a relevant loss of such protein from the nucleus (Neumann *et al.*, 2006). This sequestration would be predicted to disrupt the regulation of transcription and splicing. The identification of mutations (although rare) in the TARDBP gene in chromosome 1 which resulted in increased fragmentation and toxicity to neurons, strongly supports a patho-physiological role for TDP-43 misaccumulation in ALS (Sreedharan *et al.*, 2008; Buratti *et al.*, 2005; Abhyankar *et al.*, 2007).

VAPB

A point mutation (P56S) in the vapb gene encoding an endoplasmic reticulum (ER)-integrated membrane protein [vesicle-associated membrane protein-associated protein B (VAPB)] causes autosomal-dominant ALS (ALS8; Nishimura *et al.*, 2004). A pathogenic mechanism for this mutation has been suggested since the total loss of VAPB function in cellular response to unfolded protein, induced by one P56S mutant allele, could account for accumulation of unfolded and misfolded proteins in ER (Ron & Walter 2007; Suzuki *et al.*, 2009).

Neuroinflammatory responses

One event, which has been proposed to contribute to the selective motor neuron degeneration in ALS, is the altered immune response and inflammation reaction (Appel *et al.* 1995, Poloni *et al.* 2000, Yi *et al.* 2000, Cereda *et al.* 2008). The autoimmunity implication was suggested by the presence of antibodies against voltage-gated calcium channels in the sporadic forms, which lead to impairment of neuronal intracellular calcium homeostasis (Appel *et al.* 1995). The anti-Fas auto-antibodies, which induce apoptosis in neuronal cultures (Yi *et al.* 2000), were also found in sera of ALS patients (Sengun & Appel 2003).

Glia-mediated neuroinflammation seems to play an important role among the mechanisms of neurodegeneration. Inflammatory cytokines have been implicated in many CNS disorders with an inflammatory component (Toulmond *et al.* 1996). Although contrasting results have been reported for cytokine levels in the CSF (Almer *et al.* 2002) or plasma of ALS patients, increased concentrations of IL-6, TNFα and MCP-1 suggest a neuroinflammatory contribution to the etiological pattern of the disease (Baron *et al.* 2005, Cereda *et al.* 2008, Ford & Rowe 2004, Moreau *et al.* 2005).

In particular, TNFα is an important mediator of inflammatory and immune processes and a TNF signalling pathway has been demonstrated to mediate both apoptotic and necrotic cell death (Wallach *et al.* 1999). Furthermore, TNF-α is up-regulated in neurodegenerative disorders, such as multiple sclerosis, Parkinson's and Alzheimer's diseases (Deigner *et al.* 2000, Lue *et al.* 2001) and high circulating levels of TNF and its soluble receptors were found in the blood of ALS patients (Cereda *et al.* 2008, Poloni *et al.* 2000), thus suggesting a possible involvement of this cytokine in the pathogenesis of motor neuron degeneration. TNFα can also induce astrocytes to produce interleukin 8 (IL-8, Kasahara *et al.* 1991), another pro-inflammatory cytokine which has been shown to be toxic for neurons (Maini *et al.* 1995). Since TNF-α has been shown to strengthen the glutamate-mediated neurotoxicity in human foetal neuronal cultures (Chao & Hu 1994) and injection of kainic acid increases the level of TNF-α mRNA in rat brain (Minami *et al.* 1991), the interactions between TNF signalling and excitotoxic injuries could also represent a relevant contribution to motor neuron degeneration (Ghezzi & Mennini 2001).

1.6 Therapy and patient's care

Many therapeutic strategies for ALS have been devised and tested in clinical trials over the years (Forbes *et al.* 2004, Liebetanz *et al.* 2004, Preux *et al.* 1996, Scarmeas *et al.* 2002), but as yet, the only agent that has been shown to confer improved survival in ALS is riluzole (Rilutek®; Aventis Pharma SA, France; (Bensimon *et al.* 1994, Miller et al. 2007). The benefit is modest, prolonging life for an average of about 3 months if the drug is taken for 18 months. One of the actions of riluzole is the inhibition of glutamate-release by acting on sodium channels. Findings of an initial trial in individuals with this disorder suggested that riluzole was more effective in bulbar-onset than limb-onset patients (Lacomblez *et al.* 1996). This result was not repeated in a

second, larger, dose-ranging trial (Miller *et al.* 2007). Further studies were recommended to investigate aspects of the potential effectiveness of riluzole in amyotrophic lateral sclerosis. Riluzole, indeed, does not represent a cure, or even a very effective treatment, and the search for better therapeutic agents continues. But clinical trials in ALS are difficult and expensive to perform. Trials must involve many clinical centres to allow enrolment of sufficient numbers for statistical validity. Recent advances in patient care, such as assisted ventilation via nasal intermittent positive-pressure ventilation, care in multidisciplinary teams and tube feeding have improved patient's survival so that it is very difficult to compare previous drug-dependent benefits to the contemporary results. The main currently recommended interventions for the cure and care of ALS patients are reported in the diagram in Fig. 1.6.1.

There is still no reliable surrogate marker for early disease in ALS. Most researchers believe that therapeutic agents will be of most benefit if given early in the disease. Various neuraxis imaging modalities, magnetic resonance imaging (MRI), diffusion MRI, functional MRI, and single photon emission computed tomography (SPECT) have been examined, but no specific and sensitive imaging features to allow early diagnosis have been identified (Kalra & Arnold 2003). Likewise, no serum or CSF markers of early disease have yet been found (Shaw & Williams 2000).

Many of the agents undergoing clinical trials in ALS have shown good effects in the mSOD1 mouse model, both in reducing the rate of disease progression and in prolonging survival. However, the benefits in the mouse have translated into clinical efficacy only in the case of riluzole. Possible reasons for this have recently been reviewed (Rothstein 2003) and include (1) difficulties in extrapolating equivalent dosesfrom mouse to man, (2) species differences in the anatomy of the motor system, the permeability of the blood–brain barrier, the ratio of neurons to glial cells and the

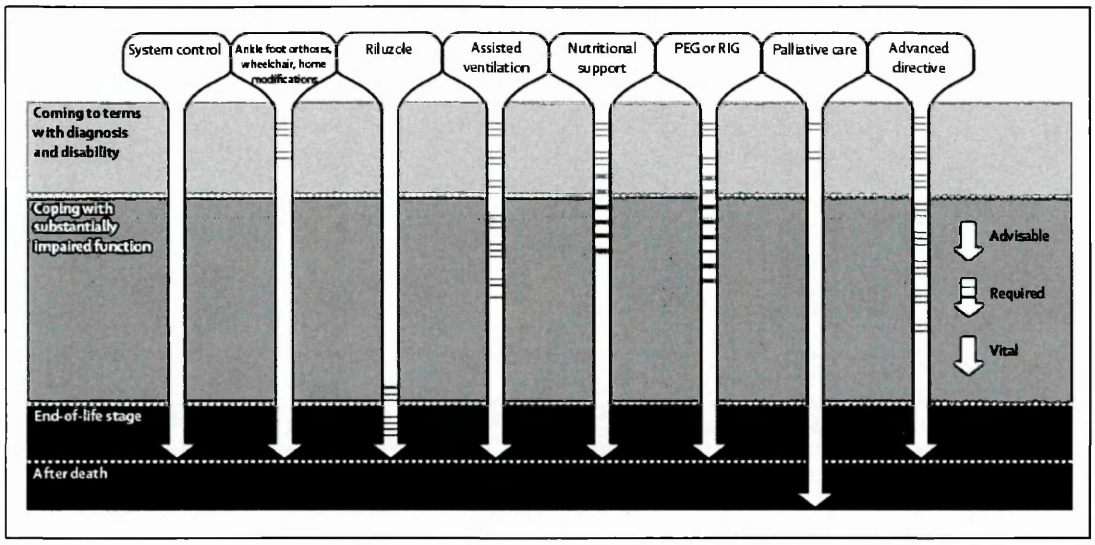


Fig. 1.6.1 Recommended strategy for ALS patient's care.

PEG= percoutaneous endoscopic gastrostomy. RIG=radiologically inserted gastrostomy (modified from Radunovic *et al.* 2007).

immune response, and (3) the fact that the mSOD1 rodent models involve huge overexpression of the human mSOD1 gene (17-fold overexpression in the G85R mSOD1 mouse and 40-fold overexpression in the G93R mouse according to Jonsson *et al.* 2006) compared with the single gene dose effect in human mSOD1- mediated fALS. There is clearly need for developing further animal models that more closely resemble the human disease. Many of the drug trials in the mouse model reported better results when agents were administered early, before the mice develop clinically apparent weakness, which is of questionable relevance in human ALS in the absence of an early identified surrogate marker for the disease. Some of the compounds assessed in completed and ongoing clinical trials are listed in Fig1.6.2.

Trials of cocktails of therapies – combining agents that act on different proposed mechanisms, such as minocycline, riluzole and nimodipine – have given excellent results in the mouse model (Kriz *et al.* 2003). It seems likely that successful therapy in

ALS will similarly involve a mix of agents, acting synergistically on various mechanistic targets to interrupt the final common pathway of motor neuron degeneration. In the last decade the rationale to define a therapy for ALS was to plan clinical trials by agents that: 1) have been shown to be effective in *in vitro* or *in vivo* models of motor neuron degeneration, such as neurotrophic agents, compounds that support mitochondrial activity, or anti-apoptotic agents; 2) have been reported to counteract the detrimental effect caused by stimuli that have been postulated to be involved in ALS, such as antiglutamatergic agents, anti-inflammatory agents, calcium regulators and antiviral drugs.

Drug	Mechanism	Dose and route	Trial design	Status/outcome
AEOL-10150	Antioxidant	Dose not known, subcutaneous	Not known	Phase I clinical trial status: pivotal phase II/III trials planned
Brain-derived neurotrophic factor (BDNF)	Neurotrophin	25 or 100 µg/kg	Randomised, placebo-controlled, dose-ranging trial	Failed to show benefit for primary endpoints
		25, 60, 150, 400, or 1000 µg/day, intrathecal	Randomised, double-blind, sequential, dose-escalation study	Intrathecal BDNF well tolerated in doses of up to 150 µg/day; few patients did not permit conclusions on effectiveness
Ciliary neurotrophic factor (CNTF)	Neurotrophin	0·5, 2, or 5 µg/kg per day	Double-blind, placebo-controlled, dose-ranging trial	No beneficial effect on progression; adverse events and deaths increased in 5 µg/kg group
		15 or 30 µg/kg, subcutaneous	Randomised, placebo-controlled, dose-ranging, double-blind trial	No significant difference between CNTF and placebo; side-effects sufficient to limit use in many cases
Creatine	Stabilise mitochondrial function	Creatine monohydrate 10 g/day	Double-blind, placebo-controlled, sequential trial	Did not have a beneficial effect on survival or disease progression
Gabapentin	Antiexcitotoxic agent	3600 mg/day	Randomised, placebo-controlled trial	No evidence of beneficial effect on disease progression or symptoms
Glatiramer acetate	Immunosuppressant	..	Randomised controlled trial	Status uncertain; further studies needed
Interferon beta 1A	Immunomodulatory agent	12 mIU, subcutaneously, three times a week	Randomised, placebo-controlled trial	The results of this pilot study suggest that interferon beta 1A is not effective
Lamotrigine	Glutamate release inhibitor	300 mg/day, oral	Double-blind, placebo-controlled, crossover	No evidence of effectiveness
Minocycline	Inhibits glial and caspase activation	Up to 400 mg/day, oral	Compound, with and without riluzole	Status not yet clear; further trials planned
ONO 2506 (Cereact)	Astrocyte stabilising drug	1·2 g/day, oral	Compound, with and without riluzole	Results pending
Pentoxifylline	Tumour necrosis factor α inhibitor	1·2 g/day	Double-blind, randomised, placebo-controlled, multicentre trial	Not beneficial and should be avoided in patients treated with riluzole
Recombinant IGF-1	Neurotrophin	0·05 and 0·1 mg/kg per day, subcutaneous	Placebo-controlled dose ranging trial	Interpretation of trial results jeopardised by trial design, further studies in progress
Riluzole	Glutamate release inhibitor	100 mg/day, oral	Placebo-controlled dose-ranging trial	Modestly effective
Topiramate	Antiexcitotoxic agent	Up to 800 mg/day	Double-blind, placebo-controlled, multicentre, randomised clinical trial	Did not alter decline in forced vital capacity and amyotrophic lateral sclerosis functional rating scale or affect survival; further studies of topiramate up to 800 mg/day are not warranted
Vitamin E	Antioxidant	α tocopherol (500 mg twice a day)	Randomised, placebo-controlled trial	No effect on survival and motor function
Xaliproden	Enhancement of nerve growth factor gene expression	1 and 2 mg/day, oral	Random allocation to one or two doses of xaliproden or placebo; two sub-studies: one with all patients taking riluzole, the other with no patients taking riluzole	No evidence of effectiveness

Fig. 1.6.2 Disease-modifying treatments for ALS.

(taken from Goodall & Morrison 2006)

Anti-excitotoxic agents

Riluzole is, at present, the only drug approved from the Food and Drug Administration (FDA) for the treatment of ALS. Riluzole is considered to exert its protective effect in ALS patients by acting as an antiexcitotoxic agent (Lacomblez *et al.* 1996). Riluzole treatment can block different processes inducing the release of glutamate from presynaptic neurons and therefore can inhibit the glutamate-mediated overstimulation of postsynaptic glutamate receptor (Pratt *et al.* 1992, Zona *et al.* 1998). In two distinct therapeutic trials, riluzole prolonged the survival by three to six months (Bensimon *et al.* 1994, Lacomblez *et al.* 1996). In one of these trials the treatment slightly slowed the decline in the strength of limb muscle but failed to reduce others considered clinical parameters. In one retrospective analysis (Riviere *et al.* 1998) patients who received riluzole remained in a milder state of the disease longer than placebo-treated ALS patients. Further evidence, by utilizing proton density magnetic resonance spectroscopy, has demonstrated that patients treated with riluzole had a less marked progression of neuronal loss compared to the placebo treated group (Kalra *et al.* 1999). The effect of riluzole reinforced the hypothesis that glutamate-induced excitotoxicity was related to ALS and prompted the scientific community to plan several clinical trials using more specific antiglutamatergic agents.

Several other trials of agents targeted against glutamate excitotoxicity did not produce significant clinical improvement. Such agents included gabapentin, lamotrigine, dextromethorphan, L-threonine and calcium channel blockers (verapamil and nimodipine). This evidence suggests that the mechanism of action of riluzole is not exclusively related to the reduction of the glutamate-induced toxicity and that additional mechanisms may contribute to the actual effectiveness of this drug in ALS care.

Growth factors

Nerve growth factors (neurotrophins) have also been investigated as disease-modifying treatments for ALS. Neurotrophins are thought to have a key role in maintenance of neuronal viability, suggesting the hypothesis that they might be able to rescue dying motor neurons. Recombinant insulin-like nerve growth factor 1 (IGF-1) is a naturally occurring peptide with multitarget neurotrophic potential on motor neurons. Two randomised placebo-controlled trials of this peptide in patients with amyotrophic lateral sclerosis have been completed to date, which were seriously compromised by flawed trial design (Borasio *et al.* 1998, Lai *et al.* 1997). A Cochrane review (Mitchell *et al.* 2002) has been undertaken, and the effectiveness of recombinant IGF-1 for treatment of this disorder remains unproven, although the drug might be modestly effective. Hopefully, a current trial in North America will provide new insights into the effectiveness of this peptide in ALS.

Ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) have both shown promising results in vitro and in animal models. However, findings of clinical trials with parenteral and (for BDNF) intrathecal administration have been disappointing (Bongioanni *et al.* 2004, Ochs *et al.* 2000).

Anti-inflammatory and anti-apoptotic agents

Much current interest is focused on the tetracycline antibiotic minocycline, as this has been the most effective agent in prolonging life in the rodent mSOD1 model (Kriz *et al.* 2002). The exact mechanism is unclear but thought to be via inhibition of microglial activation and modulation of apoptosis (Yong *et al.* 2004). The antibiotic has good CNS penetration when taken orally, and clinical trials in various neurodegenerative disorders, including ALS, are now ongoing.

A trial of Copaxone (glatiramer acetate), widely used in the treatment of multiple sclerosis, is in progress. In the mSOD1 model, administration of the drug improved

survival by 25% via the proposed mechanisms of increased T-cell derived interferon γ and enhanced glutamate transporter expression (Angelov *et al.* 2003).

Arimoclomol is one of a novel family of 'smart drugs', co-inducing the expression of HSPs only under times of cellular stress. Treatment with arimoclomol after symptom onset in mSOD1 mice delayed disease progression and increased survival, suggesting that chaperone induction may be a good target for effective therapy in humans (Kieran *et al.* 2004). Human trials are currently ongoing in the USA.

Symptomatological and palliative care

Palliative care is aimed at relief of symptoms and improvement in quality of life for patients whose disease is not responsive to curative treatment, and for their families (World-Health-Organization 1990), although palliative care is still perceived as the last resort (terminal care). Despite first attempts at establishing evidence-based guidelines, standards of palliative treatment in patients with amyotrophic lateral sclerosis are still largely based on expert opinion (Mitsumoto *et al.* 2005, Simmons 2005) and differ between countries (Borasio *et al.* 2001). When oral food intake becomes intolerable because of choking, percutaneous endoscopic gastrostomy should be undertaken (Kasarskis *et al.* 1999). In patients with a forced vital capacity less than 50%, gastrostomy placement should be done after institution of non-invasive ventilation because of the increased risk of respiratory insufficiency (Gregory *et al.* 2002). Alternatively, a radiologically inserted gastrostomy can be considered (Chio *et al.* 2004). Dysarthria can lead to complete loss of oral communication. Speech therapy is helpful initially if progression is slow. Modern computer technology can enable even quadriplegic patients to communicate effectively. First attempts at directly exploiting brain electric currents to control computers have shown encouraging results (Kubler *et al.* 2005).

Dyspnoea can be a very distressing symptom in patients with ALS and most affected individuals die from respiratory failure. Dyspnoeic attacks usually have a pronounced anxiety component and are best managed by short-acting benzodiazepines (lorazepam). Particularly in chronic dyspnoea, the feeling of shortness of breath is best treated with morphine. Titration of the morphine dose against the clinical effect will almost never lead to life-threatening respiratory depression (Sykes & Thorns 2003). Non-invasive ventilation is an efficient and cost-effective palliative measure for nocturnal hypoventilation (Mustfa *et al.* 2006). Other symptoms of amyotrophic lateral sclerosis that can be relieved by appropriate drugs include muscle cramps, fasciculations, spasticity, and drooling.

1.7 Animal models

The development of animal models for human diseases is an important scientific goal since they can provide valuable insights into the disease process itself. A valid animal model of a human disease should recapitulate the pathogenic process, present similarity in the clinical and pathological features of human disease, and responsiveness to disease-modifying events, such as treatments.

Several animal models of ALS have been proposed but so far not one is thought to be completely satisfactory (Borchelt *et al.* 1994, Gurney 1994, Pioro & Mitsumoto 1995, Strong & Pattee 2000). This gap is due largely to the incomplete knowledge of the etiology and the pathogenesis of human ALS. Furthermore unsuccessful approaches reflect the inevitable limitation of accuracy reproducing human diseases in species with a different physiology and anatomy. Anyway, studies on animal models of motor neurodegenerative diseases contributed to the understanding of common detrimental processes that lead to motor neuron death. The animal models for motor neuron

disorders can be classified in three main categories: pharmaco-toxicological models, animals showing spontaneous motor neuron degeneration and transgenic mouse models. A short description of the most common animal models of ALS is reported below.

SOD1 transgenic mouse

The most characterized transgenic model of fALS is the transgenic mouse with Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1) mutation. Since 1993, when Rosen *et al.* reported the correlation between this mutation and a 20% of familial cases of ALS (Rosen *et al.* 1993), several lines of transgenic mice, and rats, carrying different point mutations associated to human mutations, were produced (Bruijn *et al.* 1997, Gurney 1994, Wong & Borchelt 1995).

The onset of symptoms differs among the different type of mutation; however, all are characterized by motor neuron degeneration. On the other hand, transgenic mice expressing the wild type form of human SOD1 (hSOD1wt) showed mild neuropathological alterations in lumbar motor neurons, and no neurological disorders, appearing phenotypically similar when compared to healthy mice (Jaarsma *et al.* 2000). The motor degenerative process follows a lumbar/cervical gradient and at the late stage of the disease almost all spinal motor neurons have disappeared with death commonly produced by respiratory failure. Degenerating motor neurons appeared highly vacuolized and at the end stage, the cytoplasm of motor neurons is almost empty. Filamentous inclusions immunopositive for ubiquitin and neurofilaments are present in some of the surviving neurons (Gurney *et al.* 1994).

Genetic strategies aimed at modifying the mutant SOD1 transgene induced the greatest benefits in these mice (Ralph *et al.* 2005, Raoul *et al.* 2005). Riluzole treatment only marginally affects disease outcome, but not onset (Gurney *et al.* 1996, Gurney *et al.* 1998). Viral-mediated delivery of growth factors such as IGF-1 and VEGF (Azzouz *et*

al. 2004, Bordet *et al.* 2001, Kaspar *et al.* 2003, Wang *et al.* 2002) to spinal motor neurons substantially rescues mice, suggesting that the delivery of neurotrophic factor into the CNS is a key-factor for therapy in ALS. HDAC inhibitors, anti-apoptotic, anti-inflammatory and novel anti-oxidant agents also appear effective at extending life in this model. However, none of these therapies completely arrests disease, suggesting

Neurofilament transgenic mice

Excessive accumulation of neurofilaments in the cell bodies and proximal axons of motor neurons is a major pathological hallmark during the early stages of many human motor neuron diseases. Transgenic mice with mutations in genes encoding for neurofilament proteins were produced as the first animal model of motor neuron degeneration. Transgenic mice that accumulate NF-L to approximately 4-fold the normal level in the sciatic nerve showed massive accumulations of neurofilaments in the motor neurons of the ventral horn of the spinal cord, swollen perikarya, and eccentrically localized nuclei (Xu *et al.*, 1993). NF-L accumulation was accompanied by an increased frequency of axonal degeneration, proximal axon swelling, and severe skeletal muscle atrophy. Transgenic mice with a genomic fragment including the complete human NF-H gene were also generated. Human NF-H proteins were produced at levels up to 2-fold the levels of endogenous mouse NF-H protein and progressively induced neurological defects and abnormal neurofilamentous swellings in NF-H transgenics, by 3-4 months of age (Cote *et al.*, 1993). Some years later, transgenic mice overexpressing wild-type peripherin (a component of intermediate filament inclusion bodies associated with degenerating motor neurons in sALS) showed massive and selective degeneration of motor axons during aging (Beaulieu *et al.*, 1999). Remarkably, the onset of peripherin-mediated disease was precipitated by a deficiency of NF-L. In NF-L null mice, the overexpression of peripherin led to early- onset formation of

intermediate filament inclusions and to the selective death of spinal motor neurons at 6 months of age. Formation of similar peripherin inclusions in presymptomatic transgenic mice expressing a mutant form of superoxide dismutase linked to ALS was also reported (Beaulieu *et al.*, 1999).

pmn mouse

The "paralysé natural mutant" (pmn) mouse carries a recessive mutation on the chromosome 13 (Brunialti *et al.* 1995). The genetic defect in pmn mice has been localized to a missense mutation in the tubulin-specific chaperone E (Tbce) gene (Bommel et al., 2002; Martin et al., 2002). The Tbce gene encodes a protein (cofactor E) that is essential for the formation of primary tubulin and tubulin heterodimeric complexes. Isolated motor neurons from pmn mutant mice exhibit shorter axons and axonal swelling with irregularly structured tubulin and tau immunoreactivity, thus providing genetic evidence that alterations in tubulin assembly lead to retrograde degeneration of motor axons, ultimately resulting in motor neuron cell death. Neuropathological and clinical signs have an early onset (2 weeks) and a rapid progression of symptoms (Schmalbruch *et al.* 1991). Symptomatic phase begins at two weeks of age and evolves rapidly to death at around 40 days. Distal axonopathy is the most relevant sign of this pathology. Neurogenic muscle atrophy and subsequent paralysis are prominent (Kennel *et al.* 1996). In the pmn mouse both growth factors and pharmacological agents, including riluzole have been shown to reduce the progression of symptoms (Haase *et al.* 1998, Sagot *et al.* 1995). Nonetheless in pmn the mechanism of motor neuron death is not yet characterized, the evidence that Bcl2 overexpression failed to slow clinical progression and to prolong the survival, would support a non-apoptotic cell death process (Blondet *et al.* 2002).

Wasted mouse

The wasted mouse carries a recessive mutation that has been identified as a deletion of a sequence of DNA on chromosome 2 that prevents the expression of the gene coding for the translation elongation factor EG1α2 (Chambers *et al.* 1998). It is still unclear why the disruption of this protein involved in protein synthesis should produce such a specific neurological syndrome.

In the wasted mouse, neuropathological and clinical signs have an early onset, before the second week of age, and a very fast progression that leads to death within a month. Pronounced vacuolization is observed throughout the anterior horn region and in the brainstem of motor nuclei (Lutsep & Rodriguez 1989). Spinal motor neurons degenerate and are lost, whilst upper motor neurons appear to be unaffected. Wasted mice also show lymphoid hypoplasia and other immunological anomalies (Kaiserlian *et al.* 1986, Libertin *et al.* 1994).

Although many neuropathological hallmarks observed in the wasted mouse are similar to those found in human disease, the early onset of symptoms and the short time of survival make it extremely difficult to determine the possible effectiveness of pharmacological treatments.

ALS2 knockout mouse

Mutation in an ALS-related (ALS2) gene has been identified as the cause of a rare autosomal recessive form of juvenile-onset ALS, also referred to as ALS2 (Ben Hamida *et al.* 1990, Hadano *et al.* 2001, Yang *et al.* 2001). In humans, the ALS2 gene is located on chromosome 2 at position 33.2, and encodes a protein called alsin, which is produced in a wide range of normal tissues, with the highest amounts in the brain and spinal cord. Although the function of ALS2 protein in motor neurons is unclear, it may play an important role in regulating cell membrane organization and the movement of

molecules within motor neurons. Therefore, it would be expected to play a role in the development of axons and dendrites. However, it is unclear how and why loss of alsin function causes the ALS2-linked diseases. To fill this gap, four types of ALS2 knockout mice have been successfully developed and studied. They are characterized by increased susceptibility to oxidative stress and to glutamate receptor-mediated excitotoxicity (Cai *et al.* 2005), age-dependent decrease in the size and number of ventral motor axons and cerebellar Purkinje cells, astrocytosis and microglial activation in the spinal cord and brain (Hadano *et al.* 2006); significantly smaller cortical motor neurons, and in addition, marked diminution of Rab5-dependent endosome fusion activity and disturbance in endosomal transport of IGF-1 and BDNF receptors (Devon *et al.* 2006). Slowed movement without muscle weakness and progressive axonal degeneration in the lateral spinal cord have also been shown (Yamanaka *et al.* 2006). Significantly, all four of these ALS2 knockout murine models show no human ALS2-like symptoms and are not neurologically analogous to patients with the ALS2 mutation.

Mutant dynactin mouse model

Dynein/dynactin is a motor protein complex required for fast retrograde transport along microtubules (Ateh *et al.* 2008, Schroer 2004). The disruption of this complex results in motor neuron disease in mice (LaMonte *et al.* 2002) and impairs clearance of aggregate prone proteins by autophagy (Ravikumar *et al.* 2005), which is a lysosomal pathway for degrading damaged organelles and aggregated proteins inside the cell.

Supporting the hypothesis that impairment of retrograde axonal transport causes motor neuron death, point mutations of the p150 subunit of the dynactin gene have been reported in ALS patients (Munch *et al.* 2004). Experimentally, on the basis of this retrograde axonal transport impairment theory, mice overexpressing dynamitin,

which is a subunit of dynactin, have been produced, and show disruption of the dynein/dynactin complex, leading to inhibition of retrograde axonal transport. Lines of mutant p150 ^{glued} mice exhibit a rapid disease progression indicating that this mouse model closely mimics the clinical outcomes of sALS. Moreover, these mice share important pathophysiological alterations with the human pathology, including loss of motor neurons, ubiquitin-positive inclusions, accumulation of neurofilaments and astrogliosis (Laird *et al.* 2008).

Wobbler mouse

The wobbler mouse is one of the best characterized model of spontaneous motor neuron degeneration (Falconer 1956). The mutated gene responsible for the disease is autosomal recessive and associated with a missense mutation L967Q (Schmitt-John *et al.* 2005) in a Vacuolar–vesicular Protein Sorting (Vps54) involved in the tethering of vesicles retrieved between late endosomes and the Golgi apparatus (Liewen *et al.* 2005). Wobbler mice show early-onset selective motor neuron death in the cervical spinal cord (reviewed in Beghi & Mennini 2004). The onset of symptoms and their progression coincide with the rate of motor neuron loss. Glial activation (Bigini *et al.* 2001, Boillee *et al.* 2001, Rathke-Hartlieb *et al.* 1999) and upregulation of TNF-α (Schlomann *et al.* 2000) have been reported in the cervical spinal cord of pre-symptomatic or early-symptomatic mice. Wobbler mice show a progressive atrophy of foreleg muscles accompanied by a marked decrease in muscular strength and motor ability. Although the wobbler disease was classified for a long time in the spinomuscular atrophy (SMA) group because no neuronal alterations were detected in the cortex, this has been refuted using in vivo proton magnetic resonance spectroscopy. In this study Pioro observed that the cell bodies and the neurites of neurons of the neocortex had a strong immunoreactivity for ubiquitin and an accumulation of NF-H was observed in neuronal-

cell bodies of the cerebral cortex (Pioro *et al.* 1998). Although the actual loss of neurons has not yet been demonstrated in the cortex, this result suggests that the neuronal target in wobbler mice is not restricted to lower motor neurons but may involve other brain areas. This finding renders this model more similar to ALS than to SMA (Pioro *et al.* 1998).

The wobbler mouse is a reliable model to investigate the symptomatological, neuropathological and biochemical alterations leading to motor neuron degeneration. The main etiopathological hypotheses that have been considered responsible for motor neuron death in humans, such as excitotoxicity, oxidative stress, deficit of trophic factors and accumulation of toxic proteins and aggregates, have been tested in wobbler mice. The results obtained have pointed out several defects that are common to the human disease.

The rapid progression of symptoms and the possibility to easily score the evolution of motor impairment make the wobbler mouse a valid tool to evaluate the efficacy of different pharmacological treatments. Several agents tested during recent years showed beneficial effects by reducing clinical progression and/or motor neuron loss. These include growth factors (Ishiyama *et al.* 2002, Mitsumoto *et al.* 2001) and cytokines (Ikeda *et al.* 1995a) as well as antioxidant (Abe *et al.* 1997, Ikeda *et al.* 1995b) or mitochondria-targeted (Ikeda *et al.* 2000) pharmacological treatments. Treatment with a TNF-binding protein (rhTBP-1) delayed both symptom progression and motor neuron loss (Bigini *et al.* 2008). Riluzole treatment slowed the progression of neuromuscular dysfunction and partially prevented motor neuron death in this model (Fumagalli *et al.* 2006, Ishiyama *et al.* 2004).

Many others pharmacological treatments with different types of agents, such as steroid hormones, gangliosides, plasminogen activators, were reported to be active in reducing

the disease progression and the neuropathological alterations observed in the wobbler mouse (Bose *et al.* 1999, Gonzalez Deniselle *et al.* 1997, Gonzalez Deniselle *et al.* 1999, Gonzalez Deniselle *et al.* 2002, Lisovoski *et al.* 1997, Schumacher *et al.* 2004). However, despite these encouraging results in this animal model, the clinical trials of some of these molecules had no significant effect in ALS patients.

1.8 *In vitro* models

The complexity of ALS etiology and the lack of clear results from human studies and animal models underlie the need for parallel studies on in vitro cellular models, to test specific intracellular events and hypotheses involved in the selective vulnerability of motor neurons. Several tissue culture studies using human CNS tissue have been performed, but they gave insufficient information about spinal cord neurons in culture (Gilden *et al.* 1975). Since the main limitations on obtaining human primary cultures of motor neurons are represented by the source of the tissue (the embryo), cultures of adult human spinal cord have been reported as explants or dissociated cultures (Erkman *et al.* 1989, Kim *et al.* 1988). Unfortunately, these cultures are extremely fragile and their low viability (even in basal conditions and in presence of serum and trophic factors) did not produce useful results.

Because of the difficulties in studying human spinal cord cultures, the use of non-human spinal cord cultures is considered a reliable alternative for research. Organotypic cultures are widely used in ALS research. While maintaining reciprocal cell interaction and the presence of well-differentiated motor neurons, they have two main limitations: an inability to perform analyses at the single cell level, and the detrimental effects of "experimental axotomy" on motor neurons. This form of axotomy is due to the fact that, in early postnatal age, the axons are already connected to their specific peripheral

targets, and therefore, during the preparation of organotypic slices, the proximal stump of axons remains connected to the motor neurons whereas the distal part is lost.

The possibility of performing a single cell study to avoid the problems arising from "experimental axotomy" is obtained with immortalized cell lines. Cell-lines derived from tumours, or from cells that are dedifferentiated and transformed in vitro, such as the fusion product of mouse neuroblastoma with primary mouse embryonic spinal cord neurons (Cashman *et al.* 1992) are mainly used. However the use of cell lines as a model of motor neuron degeneration has not been entirely successful. This is due mainly to the fact that these cells often appear and behave like motor neurons but they are not motor neurons. Moreover, it is important to note that they are frequently obtained by differentiation programs or from tumour cells which possess relevant differences both in terms of cell biology and in their response to different external stimuli.

For this reason, although such approaches can provide information on the basic mechanisms of cytotoxicity, primary cultures of motor neurons (obtained from embryonic rats, mice and chickens) are the most reliable and direct model to perform more accurate and rapid measurements on different cell death pathways and to evaluate the effectiveness of drug treatments.

Formation and accumulation of protein aggregates (Robertson *et al.* 2003), oxidative stress (Estevez *et al.* 1999, Peluffo *et al.* 2004, Raoul *et al.* 1999), energetic metabolism and calcium homeostasis (Ankarcrona *et al.* 1995, Kim *et al.* 2002), the role of glutamate-mediated excitotoxicity (Carriedo *et al.* 1996, Greig *et al.* 2000, Vandenberghe *et al.* 2000) have been analyzed in detail in cultured motor neurons. In vitro studies are also very important to define the metabolic interactions between astrocytes and neurons and their cytotoxic effects on motor neurons (Pellerin & Magistretti 1994, Tsacopoulos & Magistretti 1996).

Chapter 2 - Aims and objectives

Aims

A) Glutamate-mediated excitotoxicity induces motor neuron degeneration mainly by the activation of ionotropic glutamate receptors with high affinity for AMPA and kainate (Van Den Bosch *et al.* 2000). Motor neurons do express these receptors even when maintained in cultures and in particular they show the presence of calcium-permeable AMPARs which were suggested to account for the high vulnerability of this type of cell. The present study was aimed at investigating the intracellular responses induced by the activation of the AMPA receptor (NBQX-sensitive) in primary cultured motor neurons. We previously showed that after the exposure of primary motor neuron cultures to equipotent concentrations of glutamate agonists inducing about 50% of cell death after 48 hours (chronic treatment), kainate, but not AMPA or NMDA, seems to induce apoptotic motor neuron death (Comoletti *et al.* 2001). Starting from these data, we investigated the main intracellular mechanisms that are induced by AMPAR agonists. Interactions between mediators of the inflammatory signalling and the AMPAR-dependent excitotoxic pathway were studied by evaluating the intracellular effects of two important cytokines, i.e. TNF-α and IL-8.

In light of the results obtained from the study of the AMPAR-dependent pathways we planned to test the effectiveness of potentially neuroprotective drugs which could interfere with the intracellular death mechanisms of motor neurons.

B) A second aim was to investigate the role of protein aggregation, which is one of the main etiological events in ALS, in *in vitro* motor neuron degeneration. Thus, we studied the effect of α-synuclein, which is responsible for protein aggregations in different neurodegenerative disorders (Bennett 2005), on motor neuron viability. Primary cultures were exposed to the fusion protein TAT-α-syn, derived by fusion of α-

synuclein and the TAT sequence of HIV, which is membrane permeable and can be internalized inside motor neurons, resulting in intracellular accumulation.

Objectives

The main objectives of this study were:

1) to set up and standardize a useful in vitro model to study motor neuron features and responses to excitotoxic stimuli
2) to investigate the intracellular responses to treatment with AMPAR agonists
3) to study the possible contribution of mediators of neuroinflammation to motor neuron death which were correlated with excitotoxicity
4) to analyze the effect of intracellular aggregations of α-synuclein on motor neuron viability
5) to test potentially neuroprotective drugs interacting with the degenerative pathways

1) There are many difficulties in obtaining long term primary cultures with healthy and numerous motor neurons, in particular when purified cultures are needed in order to avoid the interaction of glial cells. Since motor neuron cultures can be obtained and maintained with different procedures (animal sources, purification processes, culture media, in vitro ageing, etc.), and the cellular sensitivity to exogenous stimuli (i.e. responses to pharmacological treatments) are definitely dependent on culture conditions, direct comparisons between the results reported by different authors can be hardly done. Starting from the original work of Camu and Henderson (Camu & Henderson 1992), many groups used a metrizamide gradient and/or the immunopanning method to obtain purified motor neurons from dissociated spinal cord extracts (Greig *et al.* 2000, Herreros *et al.* 2000, Vandenberghe *et al.* 1998, Vargas *et al.* 2006), but this reagent is

no longer available and alternative methods of purification have been reported. In particular, the density gradient medium OptiPrep, a iodixanol solution, has been successfully used to obtain motor neuron-enriched cell fractions (Duong *et al.* 1999, Haastert *et al.* 2005, Misgeld *et al.* 2005). However, in these papers no attempt to obtain a purified glial fraction was reported. In the present study we set up a new procedure for the concomitant purification of motor neuron and glial fractions from the anterior horns of mouse embryo spinal cord based on the OptiPrep gradient, and we directly compare different types of primary motor neuron cultures (mixed anterior horn cultures, purified motor neuron cultures and cocultures of purified motor neurons seeded on a mature glial layer). The morphological features of motor neurons maintained under the three different culture conditions were studied by evaluating the axonal outgrowth and the perimeter and area of the somata of SMI32-positive motor neurons. Furthermore, the effect of AMPAR activation induced by the agonists kainate and AMPA, considered to play an important role in ALS etiology (Carriedo *et al.* 1996, Jahn *et al.* 2006, Mennini 2004, Van Den Bosch *et al.* 2000), was investigated. Immunocytochemical assays were used to define the cellular death kinetics induced by AMPAR agonists. This enabled the conditions (time of exposure, agonist concentration, type of culture, etc.) to be set for the analysis of the intracellular events and to define the different drug-dependent death rates. The death kinetics of the AMPAR agonists in mixed anterior horn cultures and cocultures were obtained by detecting motor neuron viability after treatments.

2) The degenerative pathways induced by excitotoxic insults were studied with cytochemical, immunocytochemical and biochemical assays and analyzed by immunofluorescent microscopic methods and microphotography techniques.
Some of the fundamental events involved in the apoptotic process were evaluated: DNA fragmentation (detected by a DNA binding dye), the expression of phosphatidylserine

residues on the membrane's outer leaflet (revealed by the annexin-V binding) and the activation of secondary apoptotic markers at early stages of motor neuron degeneration, such as procaspase and caspase proteins (with specific antibodies against the activated forms). Further intracellular degenerative events were analysed by studying the mitochondrial involvement and the mitochondrial-dependent signalling. The reduction of the mitochondrial transmembrane potential ($\Delta\Psi M$) is an upstream apoptotic event and is determinant for the release of the apoptotic initiating factors (AIFs), among which cytochrome *c* has a pivotal role (Marchetti *et al.* 1996, Susin *et al.* 1997). The detection of cytochrome *c* release was revealed using specific antibodies.
Subsequently the role of calcium intracellular influx in motor neuron degeneration was studied. The levels of intracellular free calcium and mitochondrial calcium will be detected after different excitotoxic insult exposures by using different fluorescent dyes for cytosolic or mitochondrial calcium.

3) Since TNF-α has been shown to strengthen the glutamate-mediated neurotoxicity in human foetal neuronal cultures (Chao & Hu 1994) and injection of kainic acid increases the level of TNF-α mRNA in rat brain (Minami *et al.* 1991), the interactions between TNF signalling and excitotoxic injuries could represent a relevant contribution to motor neuron degeneration (Ghezzi & Mennini 2001). Thus, we planned to test the effect of TNF-α on motor neuron viability in different culture conditions. This would also provide information about the contribution of glial cells.
TNF can also induce IL-8, another pro-inflammatory cytokine which has been shown to be toxic for neurons (Brennan *et al.* 1995, Maini *et al.* 1995). The IL-8 receptor CXCR2 is the most strongly expressed chemokine receptor on neurons and it is strongly upregulated in neuritic plaques in Alzheimer's disease (Horuk *et al.* 1997, Xia & Hyman 2002). Interestingly, CXCR2 and AMPAR were found to be co-expressed in

several CNS regions (Bigge 1999, Giovannelli *et al.* 1998) and CXCR2 activation through its ligand MIP-2 was demonstrated to enhance the amplitude of the spontaneous AMPAR-mediated excitatory activity in rat cerebellar slices (Lax *et al.* 2002). Starting from this evidence, the effects mediated by TNF receptors (TNFR1/2) or by the IL-8 receptor (CXCR2) activation and their interactions with the AMPAR-mediated motor neuron degeneration were investigated.

4) On the basis of the evidence suggesting the involvement of α-synuclein in the pathogenesis of ALS, both by human and animal studies, we investigated the effect of the protein in purified cultured mouse motor neurons to clarify the role of α-synuclein in motor neuron degeneration. In particular, we focused on the possible neuroprotective or neurotoxic roles of the protein in our cellular model of neurodegeneration. To achieve this point a-synuclein was introduced in motor neurons by incubating cultures with the fusion protein TAT-a-synuclein. This was generated from the insertion of the sequence containing the minimal translocation domain of the HIV1 protein TAT in frame before the N-terminal of the corresponding a-synuclein cDNA. α-Synuclein internalization and its effect on motor neuron viability was then investigated by immunochemistry and immunofluorescence using the primary antibody anti-α-synuclein mAb (Transduction Laboratories, Lexington, KY).

5) The identification of the intracellular mechanisms activated by AMPAR agonists could suggest a potential pharmacological approach aimed at interfering with different targets of the degenerative pathways. Thus we studied potential neuroprotective approaches on cultured motor neurons which were activated to die by excitotoxic stimuli or by inflammatory mediators.

EPO and its derivatives

Erythropoietin (EPO) is a glycoprotein originally identified as the regulator of erythroid progenitor cells. EPO is induced in hypoxic conditions through the HIF-1 transcription factor (Semenza & Wang 1992). Different findings indicate that systemically-administered EPO crosses the blood brain barrier (Brines *et al.* 2000) and is neuroprotective in animal models of brain and spinal cord trauma, and ischemia (Cerami *et al.* 2002) and retinal ischemia (Junk *et al.* 2002). Thus, in addition to promoting the hematopoietic effect, EPO has protective effects in different in vitro and in vivo models of neurodegeneration, although the molecular mechanisms involved in this EPO activity are still not fully understood (for review see Chong *et al.* 2002). Starting from the evidence that EPO could prevent the death of primary cultured motor neurons exposed to kainate or serum deprivation (Siren *et al.* 2001) we investigated its effect on motor neuron degeneration induced by different AMPAR agonists.
Because chronic administration of EPO results in an increase in the hematocrit, which could have undesirable effects, for instance by increasing the risk of thrombosis, different nonerythropoietic molecules derived from EPO have been designed that retain the neuroprotective activities of EPO. One of these molecules, carbamylated EPO (CEPO), has proven effective in animal models of stroke, EAE, spinal cord injury, and diabetic neuropathy (Leist *et al.* 2004). Unlike EPO, CEPO does not bind to the classical homodimeric EPO receptor (EPOR) (Leist *et al.* 2004), and its neuroprotective action appears to require a common sequence (common β chain) of IL-3/IL-5/GM-CSF receptor (also known as CD131) (Brines *et al.* 2004), which can functionally associate with EPOR (Jubinsky *et al.* 1997). Another nonerythropoietic EPO derivative is asialo erythropoietin (ASIALO-EPO), which, although it binds to the classic homodimeric EPOR, has a short half-life in vivo and does not increase the hematocrit (an activity that requires persistent circulating levels of EPO) but also retains neuroprotective activities

in vivo (Erbayraktar *et al.* 2003). An interesting approach to avoid the erythropoietic activity of EPO was the use of HBP, a synthetic peptide containing the amino acid sequence corresponding to helix B (residues 58–82) region of EPO, which is needed for the binding to CD131. HBP lacks the regions of EPO that interact with EPOR.

In the present study, we planned to verify the effect of CEPO, ASIALO-EPO or HBP on motor neuron viability and their potential neuroprotective activity against AMPAR-mediated toxicity. This allowed the testing of new pharmacological approaches which might exert protection of motor neurons without inducing undesirable effects.

Reparixin

Reparixin, an orally active CXCR1/2 inhibitor, reduces PMN infiltration and has protective activities in rat models of cerebral ischemia, (Garau *et al.* 2005, Villa *et al.* 2007) and it is currently being tested in a phase 2 clinical trial for graft dysfunction after kidney or lung transplantation. CXCR2 is a chemokine receptor which mediates the effect of IL-8 (called MIP-2) in rodents. It is expressed in neurons and was suggested to have pathophysiological role in neurodegenerative diseases, like Alzheimer's disease (Horuk *et al.* 1997, Xia & Hyman 2002).

The finding by Gorio and colleagues (Gorio *et al.* 2007) on the efficacy of reparixin in reducing MIP-2 concentrations, oligodendrocytes apoptosis and finally the axon demyelination in an in vivo model of spinal cord injury supports the possible neuroprotective role of CXCR2 inhibitors in the spinal cord. Since we found that MIP-2 induced neurotoxicity in cultures, we tested the efficacy of reparixin in preventing motor neuron death.

MATERIALS & METHODS

Chapter 3 - Materials

Reagents and materials that were utilized during this thesis are listed below:

- 2-mercaptoethanol, SIGMA, Italy
- ABC complex, Vector Laboratories, UK
- AMPA, Tocris, Italy
- Anti-α-synuclein monoclonal antibody (MAb), Transduction Laboratories, US
- Anti-Active capase 3 antibody, Promega, Italy
- Anti-Active caspase 9 antibody, Alexis Biochemical, UK
- Anti-annexin V polyclonal antibody (sc-8300), Santa Cruz Biotechnology Inc., US
- Anti-cytochrome *c* antibody, BP Biosciences, Italy
- Anti-EPOR polyclonal antibody (sc-5624, against the N-terminus residue of human EPOR) and blocking peptide, Santa Cruz Biotechnology, US
- Anti-glial fibrillary acid protein (GFAP) monoclonal antibody, Immunological Science, Italy
- Anti-GluR1/2/3/4 antibodies, Immunological Science, Italy
- Anti-IL-8RA (sc-23811), anti-IL-8RB (sc-683) polyclonal antibodies and their blocking peptides, Santa Cruz Biotechnology, US
- Antimicin A, SIGMA, Italy
- Anti-oligomer polyclonal antibody (A11), BioSource Europe, Belgium
- Anti-TNFR1 antibody, Hycult Biotechnology, The Netherlands
- Anti-TNFR2 antibody, Hycult Biotechnology, The Netherlands
- AraC, SIGMA, Italy
- B27, Invitrogen, Italy
- BDNF, Amgen, US

- Biotinylated anti-mouse antibody, Vector Laboratories Inc.,UK
- BSA, SIGMA, Italy
- calcium green, CG5AM, fluorescent dye for cytosolic and nuclear calcium, Molecular Probes, Eugene, OR, US
- CD131: anti-IL-3/R β (sc 679) polyclonal antibody (raised against a peptide mapping at the N-terminus of the mouse IL-3 receptor β chain), Santa Cruz Biotechnology, US
- CPW399, synthetized by Prof. G. Campiani, University of Siena, Italy (Campiani *et al.* 2001)
- Cyclosporin A, SIGMA, Italy
- Diaminobenzidine, SIGMA, Italy
- DMEM, Invitrogen, Italy
- DMSO, SIGMA, Italy
- EDTA, Fluka Biochemical, Germany
- F12 nutrient mix, Invitrogen, Italy
- FBS, Invitrogen, Italy
- FCS, Life Technologies, US
- Fluorescent FITC- (Alexa 488) or TRITC- (Alexa 546) conjugated secondary antibodies, Molecular Probes, US
- Fura-2 AM calcium indicatror, Calbiochem, Merck KGaA, Darmstadt, Germany
- GraphPad Prism version 5.01, GraphPad Software, US
- HEPES buffer solution, Invitrogen, Italy
- Hoechst 33258, SIGMA, Italy
- Horse serum, Invitrogen, Italy
- Image Tool Software (UTHSCASA), US
- JC-1, Molecular Probes, Eugene, OR, US

- Kainic acid, Tocris, Italy
- L-glutamic acid, SIGMA, Italy
- L-glutamine, SIGMA, Italy
- NBQX, Tocris, Italy
- Neurobasal Medium, Invitrogen, Italy
- NGS, Vector Laboratories, Germany
- Olympus Fluoview Laser scanning, Japan
- OptiPrepTM was from Life Technologies Italia Srl, Milanese
- Paraformaldehyde, Merck, Darmstadt, Germany
- PBS, Invitrogen, Italy
- Penicillin/streptomycin solution, SIGMA, Italy
- Poly-L-lysine, SIGMA, Italy
- Propidium iodide, Molecular Probes, Eugene, OR, US
- Recombinant rat CXCL2/MIP-2, PeproTech, US
- Reparixin and DF1726A, Dompé pha.r.ma. s.p.a., Italy
- RHODD1, fluorescent dye for mitochondrial calcium, Molecular Probes, Eugene, OR, US
- SMI-32, Sternberger Monoclonals Inc., US
- SYTO 59, Molecular Probes, US
- Thioflavin-T, SIGMA, Italy
- Trypsin, SIGMA, Italy

Chapter 4 - Methods

4.1 Cell cultures

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that comply with National (D.L. n. 116, G.U., suppl. 40, February 18, 1992) and International Laws and Policies (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996). Primary cultures were obtained from the ventral horns of the spinal cords of 13-day-old C57 BL/6N mouse embryos or 14-day-old Sprague-Dawley rat embryos (Charles River, Calco, Italy). Dompé pha.r.ma. s.p.a. (L'Aquila, Italy) supplied 13-day-old wild-type or CXCR2-deficient (BALB/c-Cmkar2) mice.

Mixed anterior horn cultures

Spinal cords were dissected with microscopic surgical operations and meninges and other tissues (dorsal horns) were removed. The ventral horns were trypsinized and centrifuged through a BSA cushion (4%) for 10 min at 400×g. Cells obtained at this step represent mixed neuron/glia population and were seeded at a density of 50,000 cells/cm^2 into 24-mm diameter well plates on glass cover slips pre-coated with polyl-lysine and containing complete culture medium as follows: neurobasal medium, B27 (2%), 0.5 mM l-glutamine, horse serum (2%), 25 μM 2-mercaptoethanol, 25 μM glutamate, penicillin and streptomycin (1%), and 10 ng/mL BDNF. The same medium (without glutamate) was added to the cultures on 4th and 6th day in vitro. Neurobasal medium contains physiological concentrations of Ca^{2+} (1.8 mM) and Mg^{2+} (0.8 mM).

Purified motor neuron cultures

As described for mixed cultures, the ventral horns were trypsinized and centrifuged through a BSA cushion. The pellet of cells obtained at this step was suspended and purified. The purification process of motor neurons was carried out by centrifugation of the cell suspension through 6% OptiPrep cushion. After centrifugation at 800×g for 15 min, a sharp band (motor neuron fraction) on the top of the iodixanol cushion and a pellet (glial fraction) were obtained. Fractions were re-suspended, centrifuged for 7 min at 500×g on a 4% BSA cushion and re-suspended in their respective plating media. Purified motor neurons obtained from the sharp band of the iodixanol cushion were seeded at a density of 10,000 cells/cm^2 into 24-mm well plates pre-coated with poly-l-lysine and cultured and maintained with complete culture medium as described above.

Glial layer

The glial feeder layer was prepared by plating the glial fraction, obtained from the purification process described above, at a density of 25,000 cells/cm^2 into 24-mm well plates on glass cover slips pre-coated with poly-l-lysine. Culture medium was prepared with DMEM adding 0.5mM l-glutamine, foetal bovine serum (10%), penicillin and streptomycin (1%), 3.6 mg/mL glucose, sodium bicarbonate (0.2%). Glial cultures were fed three times a week with fresh culture medium and grown at 37° C in a humidified incubator with 5% of CO_2. Glial cells proliferate and reach confluence after 3–4 weeks in vitro. At this step glial cell division was halted by the exposure to 10 μM AraC solution for 72 h and then replaced by fresh medium.

Cocultures of motor neurons and glia

To obtain cocultures, purified motor neurons from the purification step were seeded at a density of 10,000 cells/cm^2 onto a mature glial layer, pre-treated with AraC, by

replacing the medium used for glial cultures with a suspension of motor neurons in complete culture medium. Fresh medium (without glutamate) was added to the cultures on 4th and 6th day in vitro.

4.2 Drug treatments

Drugs were dissolved in complete culture medium (DMSO for NBQX) and 600 µl of drug solutions (diluted to the appropriate concentrations in complete culture medium) were added to wells, after the removal of 600 µl from the total 1 mL of medium present in each well. Complete culture medium (or DMSO for experiments with NBQX) was used as vehicle and represented the control condition. Inhibition studies with antagonists (NBQX for AMPAR, reparixin for CXCR2) or co-treatment with neuroprotective drugs (EPO and its derivatives) were performed by treating cultures with a solution containing the different agents.

α-Synuclein treatment

On the seventh day in vitro (DIV 7) purified motor neuron cultures were incubated for 18h with the fusion protein TAT-α-synuclein, generated from the insertion of the sequence containing the minimal translocation domain of the HIV1 protein TAT in frame before the N-terminal of the corresponding α-synuclein cDNA (Albani *et al.* 2004). α-Synuclein internalization was then revealed by immunochemistry and immunofluorescence assays using the primary antibody anti-α-synuclein or anti oligomer A11 or by the thioflavin-T staining. To investigate the effect of α-synuclein against H_2O_2 and kainate induced toxicity, on DIV 7 motor neurons were pre-incubated with TAT-α-synuclein at the proper concentration for no less than 3h and then co-

treated with TAT-α-synuclein and 100 μM H_2O_2 or 50 μM kainate for 18 and 48 h respectively. For serum deprivation, cultures were incubated for 18 h with the medium free of serum and growth factors. The same medium was used to dilute α-synuclein when co-treatment was needed.

Motor neuron viability

The viability of motor neurons was assayed as follows: only the SMI32-positive cells, with typical morphology (triangular shape, single well defined axon), large bodies (>20μm) and with intact axons and dendrites were counted, at a magnification of 200×, following the length of the cover slip in four non-overlapping pathways. This number was compared to the mean of SMI32-positive cells counted in control wells. In a typical experiment, the number of counted SMI32-positive cells in control wells was 30 ± 7 (n = 6) in purified cultures, 45 ± 9 (n = 12) in mixed anterior horn cultures or 70 ± 11 (n = 12) in cocultures.

4.3 Cytochemical staining

Cell cultures underwent common process of cell fixation and permeabilization when not differently specified: cells were incubated with paraformaldehyde 4% (w/v) in PBS for 40 min, permeabilized with Triton X-100 (0.2%) for 30 min and blocked with FCS 10% (v/v) in PBS.

SMI 32 staining

The SMI32 antibody specifically stains the non-phosphorylated neurofilaments.. Incubation with the primary antibody SMI32 (1: 6000) was carried out overnight in blocking solution. Cells were washed and incubated with an anti-mouse fluorescent secondary antibody (Alexa 488/546, diluted 1: 1000) for immunofluorescent staining or

with a biotinylated anti-mouse secondary antibody (diluted 1: 200) for 1 h at room temperature in a dark room. The biotinylated antibody signal was amplified with avidin and biotinylated horseradish peroxidase macromolecular complex (ABC kit method), finally revealed with diaminobenzidine (0.5 mg/mL) and H_2O_2 (6 µl/10 mL).

Double staining SMI32 / active caspase-9 or -3

Fixed and permeabilized mixed anterior horn cultures or cocultures were double-stained with the SMI32 antibody and a specific antibody for activated caspase-9 or -3. The SMI32 staining assay was performed as described above. After the incubation with the anti-mouse secondary antibody (Alexa 488), cells were rinsed three times in PBS and incubated with the primary antibodies as follows. Cells were incubated overnight at 4° C, in a solution of PBS containing an anti-rabbit polyclonal antibody raised against the cleaved form of capase-9 (1:100) or capase-3 (1:100) and FBS 1%. After the incubation with the primary antibody and three rinses in PBS at RT, cells were incubated for 2 h at RT in a solution of PBS containing an Alexa-546 goat anti-rabbit secondary antibody (1:1000 for caspase-9 staining or 1:500 for caspase-3) and FBS (2%). For the detection of activated caspase-9 after 2 or 4 h treatments with 1µM AMPA the FITC-conjugated secondary antibody was used, being the SMI32 revealed by the Alexa-546 antibody.

Double staining annexin V / SMI32

For the annexin V staining, cultures did not undergo the permeabilization step. Cells were fixed by incubation with 4% (w/v) paraformaldehyde, rinsed three times in PBS pH 7.4, then incubated overnight with a solution of the primary antibody anti-annexin V (1:500) in PBS, with 10% of normal goat serum (NGS). After that, cells were rinsed with PBS and incubated with an Alexa-546 goat anti-rabbit secondary antibody

(1:1000) for 2 h at RT. After being rinsed with PBS, cells were permeabilized with Triton and the procedure for the staining of SMI32 was carried out as described above.

Double staining GluR2 / SMI32

For the GluR2 staining, cells were not permeabilized. After fixation by incubation with 4% (w/v) paraformaldehyde, cocultures were rinsed three times in PBS, then incubated overnight with a solution of the primary antibody anti-GluR2 (1:100) in PBS, with 10% FBS. After that, cells were rinsed with PBS and incubated with an Alexa-488 anti-rabbit secondary antibody (1:1000) for 2 h at RT. After being rinsed with PBS, cells were permeabilized with Triton and the procedure for the immune-fluorescent staining of SMI32 was performed as described above, using an Alexa-546 anti-mouse as secondary antibody.

Double staining SMI32 / CXC Receptors

Mixed anterior horn cultures were double-stained with the SMI32 antibody and the specific antibody for CXCR1 (sc-23811) or CXCR2 (sc-683). The SMI32 staining assay was done as described above. After incubation with anti-mouse secondary antibody, cells were incubated overnight at 4 ° C with the primary antibody sc-23811 (diluted 1: 100 in PBS) or sc-683 (diluted 1: 200 in PBS). To verify the specificity of the staining, parallel samples were incubated with the same solutions of primary antibodies with a fivefold excess of the blocking peptides. All the steps were conducted in a dark room. Cells were finally washed and incubated with an anti-goat (for CXCR1) or an anti-rabbit (for CXCR2) fluorescent secondary antibody (diluted 1: 200; excitation wavelength 546 nm).

Double staining SMI32 / TNF Receptors (cocultures)

The SMI32 staining assay was done as described above. After incubation with the Alexa-546 anti-mouse secondary antibody, cocultures were rinsed three times in TBS, incubated for 30 min in 10% NGS and then incubated overnight at RT with the primary rat anti-TNFR1 or anti TNFR2 antibody diluted 1:100 in TBS + 1% NGS. After incubation, cultures were washed in TBS + 10% NGS and incubated for 2 h with the Alexa- 488 anti-rat secondary antibody (diluted 1:1000).

Double staining GFAP / TNF Receptors (mixed anterior horn cultures)

Mixed anterior horn cultures were incubated overnight at 4 °C with the GFAP mouse primary antibody (1:500) diluted in PBS with 3% FBS and 0.1 % Triton X-100. After incubation cell were washed with PBS and 1% FCS and incubated with the Alexa-546 anti-mouse secondary antibody for 2 h. Then TNFRs staining was performed as described in the previous section.

EPO and CD131receptors

Purified motor neuron cultures were incubated overnight at 4 °C with rabbit polyclonal primary antibody raised against anti-Epo receptor (1:500) or anti-IL-3/R β (1:500) polyclonal antibody, in the absence or presence of an excess of relative blocking peptides (1:50). Next steps were carried out at room temperature. Cells were washed three times and then incubated for two hours in a solution of PBS containing biotinylated anti-rabbit (1:200) and FCS (1%). The following steps were identical to the SMI32 protocol (ABC kit method).

a-synuclein

Purified motor neurons were incubated overnight with the anti-α-synuclein monoclonal (1:100) or anti-oligomer (1 μg/mL) antibody diluted in PBS with 1% HS. After

washing, a proper fluorescent secondary antibody diluted 1:200 with 1% HS was added for 1h and cells were analysed by the fluorescent microscope.

GluR1-4

Cells were then incubated overnight at 4°C in a solution of PBS containing Triton X-100 (0.1%), FBS (3%) and one of the following primary antibodies:

- anti-rabbit polyclonal antibody for GluR1, diluted 1:500
- anti-mouse monoclonal antibody for GluR2, diluted 1:1000
- anti-goat polyclonal antibody for GluR3, diluted 1:1000
- anti-rabbit polyclonal for GluR-4diluted 1:200

After incubation with the primary antibody, and two rinses in PBS at room temperature, cells were incubated for 2 hours at room temperature in a solution of PBS containing FBS (2%) and Alexa-488 secondary antibody (1:1000). Images were acquired by confocal microscopy.

Hoechst 33258 staining

Cells were fixed overnight in Carnoy solution (3:1 methanol/acetic acid) and stained with Hoechst 33258 (0.1 μg/ml in PBS; excitation wavelength of 365 nm) for 1 h at room temperature, then washed 10 times, 5 min each, with distilled water, air-dried overnight and covered with a glass cover slip.

Propidium Iodide and SYTO 59 staining

Purified motor neurons were double stained with SYTO 59 (0.5 μM) and propidium iodide (PI, 10 μg/ml). SYTO 59 is a red fluorescent dye excited with the 633 line of a He/Neon laser and propidium iodide is excited at 488 nm with an Argon laser. The pseudocolour blue was chosen for SYTO 59 to distinguish between the fluorescence

emission of this dye and that of PI (red). The cells were incubated *ex vivo* at 37 °C for 20 min with the fluorescent probes. At the end of the incubation, the cells were washed and re-suspended in fresh buffer. Laser scanning confocal images were acquired with a DMS IRBE SP2 (Leach) inverted microscope.

Mito Tracker staining

The cell-permanent Mito Tracker probe contains a mild thiolreactive chloromethyl moiety that appears to be responsible for keeping the dye associated to mitochondrial penetration. To stain mitochondria, non-fixed cells were incubated with a submicromolar concentration of Mito Tracker (100 to 500 nM, depending on the intensity of the staining). Orange-Mito Tracker was previously dissolved in DMSO, to obtain a starting concentration of 1 mM, and further diluted (1:2 to 1:10) and added at a ratio of 1:1000 in the culture medium, maintained at 37 °C, to reach the nanomolar dilution required. The culture medium was then removed and substituted, for about 30 minutes, with the medium containing the Mito Tracker at 37 °C. After incubation the cells were immediately fixed. This rapid fixation is required to avoid that the Mito tracker probes, which passively diffuse across the plasma membrane and accumulate into the mitochondria, diffuse after incubation.

Cytochrome c

For cytochrome *c* immunostaining fixed cells were rinsed for three times in 0.1 M PBS pH 7.4 and then preincubated for 15 minutes at room temperature in a solution of PBS containing FBS (10%). After three rinses in PBS the cells were permeabilized in a solution of PBS containing saponin (0.1%) for 15 minutes at room temperature and then incubated overnight at room temperature in a solution of PBS containing an anti-mouse monoclonal antibody raised against cytochrome *c* (1:100) and FBS (5%) according to

the method previously described by Sanchez-Alcazar and colleagues (Sanchez-Alcazar *et al.* 2001).

Thioflavin-T

The thioflavin-T binding assay was used for the detection of amyloid aggregates. Purified motor neurons were fixed with paraformaldehyde as reported above and then incubated with 0.05% thioflavin-T solution for 10 minutes (as previously reported by (Albani *et al.* 2004). Thioflavin-T reactivity was analysed by the epifluorescent microscope at a wavelength of 488 nm.

Microscopy analysis

Pictures of double stained cells were obtained by a laser scanning microscope (Olympus Fluoview microscope BX61 with a confocal system FV500). Dual excitation was used, 488 nm (LASER Ar) and 543 (LASER He–Ne green). For the other fluorescent staining experiments, an optical epifluorescent microscope (Olympus BX51) coupled with an Olympus Camedia C-5060 digital compact camera were used. Images were analysed by the Olympus DPSoft software.

4.4 Intracellular calcium detection

For the detection of intracellular calcium levels ($[Ca^{2+}]_i$) by confocal microscopy techniques, cocultures were loaded with specific indicators for cytosolic and nuclear (5 μM CG5AM) or mitochondrial (0.5 μM RHODD1) calcium for 20 min at 37°C. Then cultures were washed and cover slips with cells were placed in a watertight chamber superfused with an isotonic buffer. Images were acquired and analysed with ImageJ, a public domain, Java-based image processing program developed at the National Institutes of Health.

For live imaging analysis , cocultures were loaded in the dark with 2.5 μM FURA-2 AM in Neurobasal medium containing 0.1% BSA and 250 μM sulfinpyrazone for 40 min at 37°C. Cultures were then washed in Neurobasal and kept in the dark for an additional 30 min to allow for complete dye deesterification. Cells were alternately illuminated at 340 and 380 nm and fluorescence was monitored at 510 nm. Calibration of the fluorescence ratios was not attempted since the present study aimed at evaluating relative changes in $[Ca^{2+}]_i$ following the activation of AMPAR by different stimuli rather than obtaining absolute values of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ variations were obtained from the ratio between fluorescence emission that resulted from emission at 340 and 380 nm (Grynkiewicz *et al.* 1985). Intracellular calcium variations after treatments were recorded by an epifluorescent microscope (IX81, Olympus) equipped with a thermostatic chamber which maintains cultures alive during the experiments and the CellR software (Olympus) for live imaging.

RESULTS

Chapter 5 - Culture characterization

Background

Primary motor neuron cultures have been used over the years to study different mechanisms of neurodegeneration implicated in ALS etiology. Mixed cultures, established as monolayer spinal cord extracts as well as cocultures of motor neurons plated over a layer of glial cells obtained from various regions of the CNS were also frequently used to define the metabolic interactions between astrocytes and neurons and their interplay in both trophic and cytotoxic condition (Pellerin & Magistretti 1994, Tsacopoulos & Magistretti 1996, Vandenberghe *et al.* 1998). Unfortunately, there are many difficulties in obtaining long-term primary cultures with healthy and numerous motor neurons, in particular when purified cultures are needed in order to avoid the interaction with glial cells. Since motor neuron cultures can be obtained and maintained with different procedures (animal sources, purification processes, culture media, in vitro ageing, etc.), and the cellular sensitivity to exogenous stimuli (i.e., responses to pharmacological treatments) are definitely dependent on culture conditions, direct comparisons between the results reported by different authors is difficult to achieve. Starting from the original work of Camu and Henderson (Camu & Henderson 1992), many groups used a metrizamide gradient and/or the immunopanning method to obtain purified motor neurons from dissociated spinal cord extracts (Arce *et al.* 1999, Greig *et al.* 2000, Herreros *et al.* 2000, Vandenberghe *et al.* 1998, Vargas *et al.* 2006), but this reagent is no longer available and alternative methods of purification have been reported. In particular, the density gradient medium OptiPrep, an iodixanol solution, has been successfully used to obtain motor neuron-enriched cell fractions

(Duong *et al.* 1999, Haastert *et al.* 2005, Misgeld *et al.* 2005). However, in these papers no attempt to obtain a purified glial fraction was reported.

In the present study, we have set up a new procedure for the concomitant purification of motor neuron and glial fractions from the anterior horns of mouse embryo spinal cord based on the OptiPrep gradient.

5.1 Motor neuron enrichment

The purification procedure, consisting of the separation of the cell suspension from the anterior horns (including both neuronal and glial cells) through 6% iodixanol cushion, gave a high purified motor neuron population. Among the entire cell population present in purified cultures (revealed by the phase contrast microscopy) 87 $\pm$ 6% (n = 20) were SMI32-positive cells with the typical morphological features of motor neurons (for representative pictures see Fig. 1). Seven days after being plated over a confluent glial layer, the purified motor neuron fraction yielded a higher number of motor neurons compared to that observed in the monolayer of mixed anterior horn cultures. In a typical experiment, the number of counted SMI32-positive cells in control (not treated) wells was 45 $\pm$ 9 (n = 12) in mixed anterior horn cultures while it increased to 70 $\pm$ 11 (n = 12) in cocultures.

5.2 Morphological features

We have directly compared different types of primary motor neuron cultures (mixed anterior horn cultures, purified motor neuron cultures and cocultures of purified motor neurons seeded on a mature glial layer) obtained at different steps of a common process of cell extraction. The morphological features of motor neurons maintained under the three different culture conditions have been studied by evaluating the axonal outgrowth and the perimeter and area of the somata of SMI32-positive motor neurons. Cells were

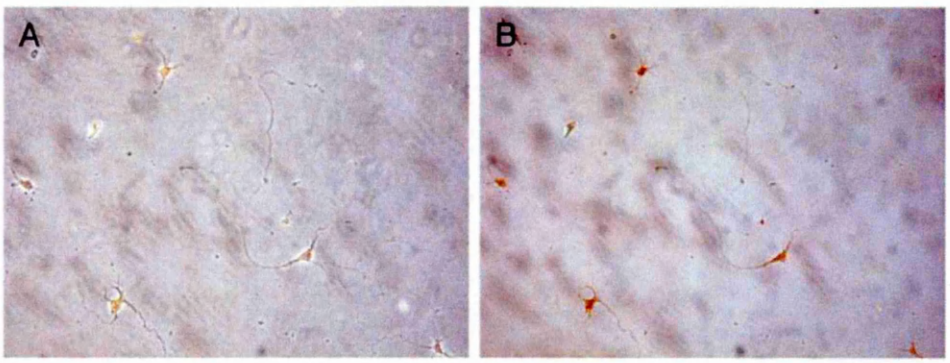


Figure 1. Representative pictures of purified motor neuron cultures. Under the experimental conditions utilized, about 90% of motor neuron purification was obtained. In these representative pictures, all the cells revealed by microscopy observation in phase contrast (A) are positive to the specific staining with SMI32 (B), having the appearance of large motor neurons. Magnification: 200X.

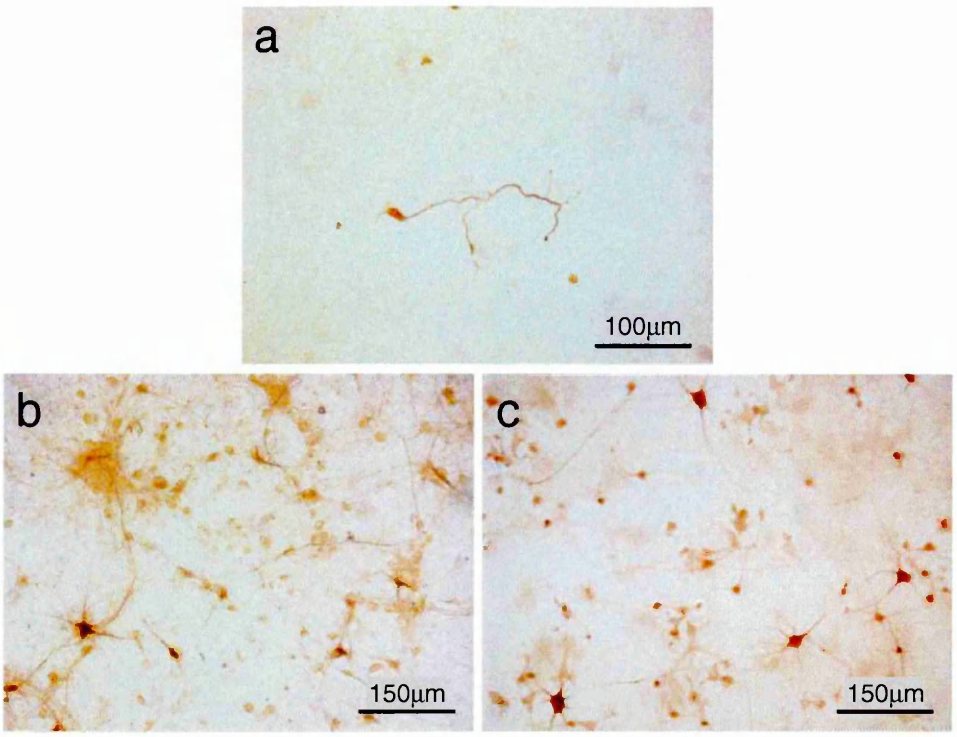


Figure 2. Primary motor neuron cultures.

Different primary cultures obtained from the ventral horns of the spinal cords of E13 mouse embryos. Motor neurons are stained with SMI32 and revealed with DAB. a) Purified motor neuron culture. b) Mixed anterior horn cultures. c) Cocultures of purified motor neurons on a glial feeder layer.

maintained in culture for 8 days, and then immunostained with SMI32 as reported above. In purified cultures the specific staining with the SMI32 antibody (Fig. 2a) and the measurement of the axonal length (Table 1) revealed low neurite outgrowth and small somata areas (Table 1) of motor neurons, even in the presence of growth factors. Mixed anterior horn cultures, obtained at the first step of cell dissociation from the ventral horns (Fig. 2b), showed a good growth and health of motor neurons as evidenced by the significant increase in axonal and somata measures (Table 1). Motor neurons grown in cocultures (Fig. 2c) showed further morphological improvements. In fact, SMI32 positive cells in cocultures had significantly longer somata perimeters, wider somata areas and increased axonal length compared both to mixed anterior horn cultures and to purified motor neuron cultures (Table 1). Furthermore, the extensive growth of purified motor neurons on mature glial layers allowed a better identification of motor neurons in cultures.

TABLE I. Quantitative morphological features of motor neurons under different culture conditions.

	Axon Length (μm)	**Somata Perimeter** (μm)	**Somata Area** (μm^2)
Purified motor neuron cultures	224 ± 33	58 ± 13	207 ± 65
Mixed anterior horn cultures	379 ± 41***	73 ± 13 *	312 ± 32***
Cocultures	937 ± 83***,$	117 ± 22 ***,$	612 ± 41***,$

Data are means ± S.D. of 15 cover slips for each condition. Five fields were analysed for each cover slip, and the mean values used for statistical analysis.

*p<0.05, ***p<0.001 different from purified cultures

$p<0.001 different from mixed neuron/glia cultures

One way ANOVA and Tukey test.

Conclusions

We have developed a valid and useful method to concomitantly separate motor neurons from glial population from the same spinal cord extract. This method showed interesting advantages:

1) the use of a commercially available compound (OptiPrep) which can substitute for metrizamide and is not toxic (at least at the concentrations used to separate cell fractions)

2) it consists of easy procedures and cheap materials

3) it allows a highly purified motor neuron fraction to be obtained

4) it is useful to establish motor neuron-enriched cocultures

Since in recent years the use of motor neuron cocultures has provided important results on the intracellular mechanisms regulating the astrocyte/neuron crosstalk and on the toxic role of different agents involved in neurodegenerative diseases, it is clear how methods aimed at providing easy, cheap and rapid tools for such investigations are extremely interesting. Using this OptiPrep-based method we were able to compare and validate results on the effect of different agents and on the role of the different cell populations in mediating them.

We directly compared, for the first time, the main morphological properties of motor neurons in three different types of cultures, all coming from the same animal source (ventral horns of the spinal cord of E13 mouse embryos) and obtained at different steps of the same purification protocol procedure. We demonstrated that motor neurons in mixed anterior horn cultures or in coculture with mature glial cells have significant improvements in axonal length and somata perimeter and area compared to those maintained in purified cultures. Moreover, motor neurons in cocultures showed significantly better values in all the morphological properties analyzed, compared

to mixed cultures. The main difference between these two culture conditions is the in vitro "ageing" of the glial population. In fact, while in mixed anterior horn cultures both the neuronal and the glial populations were cultured for 8 days in vitro, in cocultures the glial cells were maintained up to 3 weeks before the addition of motor neurons. This suggests that the long-term cultured glial layer plays a fundamental role in the health and development of cultured motor neurons, probably due to its metabolic and trophic support as well as to the intercellular signalling between the different cell populations. To provide such a neuronal support, a physical contact between glial cells and motor neurons seems to be necessary, since the soluble factors released from glia have been demonstrated not to be sufficient to induce improvement of neuronal survival or morphological differentiation (Vandenberghe *et al.* 1998).

Chapter 6 - AMPAR-mediated excitotoxicity

Background

Glutamate mediated excitotoxicity has been demonstrated to have an important role in motor neuron degeneration in ALS (Mennini 2004, Rothstein *et al.* 1995) and the AMPAR was shown to be the main receptor involved in calcium-dependent excitotoxic motor neuron death (Carriedo *et al.* 2000, Carriedo *et a*l. 1996, Van Den Bosch *et al.* 2000). Ca^{2+} overloading can be an important factor to activate intrinsic apoptotic pathways in motor neurons. The induction of apoptosis by Ca^{2+} overloading depends on the intensity of Ca^{2+} influx (Orrenius & Nicotera 1994). *In vitro* experiments on rodent cerebellar granule cells (CGC) demonstrated that high doses of glutamate produce a typical necrosis identified by the disruption of membrane integrity, the collapse of mitochondria, membrane potential decay and the swelling of nuclei. On the other hand, milder but longer lasting excitotoxic stimulation triggered a great percentage of neurons following an apoptotic pattern (Ankarcrona *et al.* 1995). These results suggest that the intensity of Ca^{2+} influx determines the selective activation of different targets, finally inducing apoptotic or necrotic/lytic neuron death (Nicotera *et al.* 1997, Ankarcrona *et al.* 1995). The typical apoptotic events were prevented both by glutamate receptor antagonists and by caspase inhibitors, while neuron death induced by high concentrations of glutamate was only weakly reduced by caspase inhibitors (Leist *et al.* 1997).

In a previous study we demonstrated that after the exposure of primary motor neuron cultures to equipotent concentrations of glutamate agonists inducing about 50% of cell death after 48 hours (chronic treatment), kainate, but not AMPA or NMDA, seems to induce apoptotic motor neuron death (Comoletti *et al.* 2001). Starting from this evidence, we studied the time-course of death induced by different AMPAR agonist concentrations and fully analysed the intracellular death mechanisms mediated by the

receptor activation under these different stimuli. In detail, we evaluated the activation of different events of the apoptotic pathway after the exposure of either mixed anterior horn cultures or cocultures to the different excitotoxins. DNA fragmentation (revealed by Hoechst33258), activation of initiator (-9) or executioner (-3) caspases, release of cytochrome *c* from mitochondria and the expression of phosphatidylserine residues on the membrane's outer leaflet (revealed by the annexin V binding) were all studied by cytological staining techniques .

The downstream event of DNA fragmentation was studied after 18 h, while the activation of caspase-9 or -3 and the annexin V binding 6 h after treatment, when the effect of the low or high AMPAR agonist concentrations on motor neuron survival was significantly different. Finally we checked the alteration in cytosolic calcium influx in motor neurons exposed to different AMPAR agonist concentrations in order to provide a detailed description of the perturbations induced by such toxic stimuli.

6.1 Time-course of AMPAR agonist-induced motor neuron death

We firstly verified whether the AMPAR subunits were expressed on motor neurons by immunocytochemical identification of the AMPAR subunits. We found that all the four subunits are widely expressed on purified motor neurons, although with different distributions (Fig. 3). GluR1 antibody showed a high immunofluorescence in the cell body, low fluorescence on dendrites, and a more intense signal in axonal terminals, especially in synaptic buttons. GluR2 showed a wide distribution on the whole cell surface, but not in the nuclear area. The subunit is moreover highly present all over the neuronal network. GluR3 showed a high localization in the somata, excluding the nuclear area. Dendrites and axons were stained with high intensity, mainly in the synaptic region. GluR4 induced high immunopositivity in the cell body and above

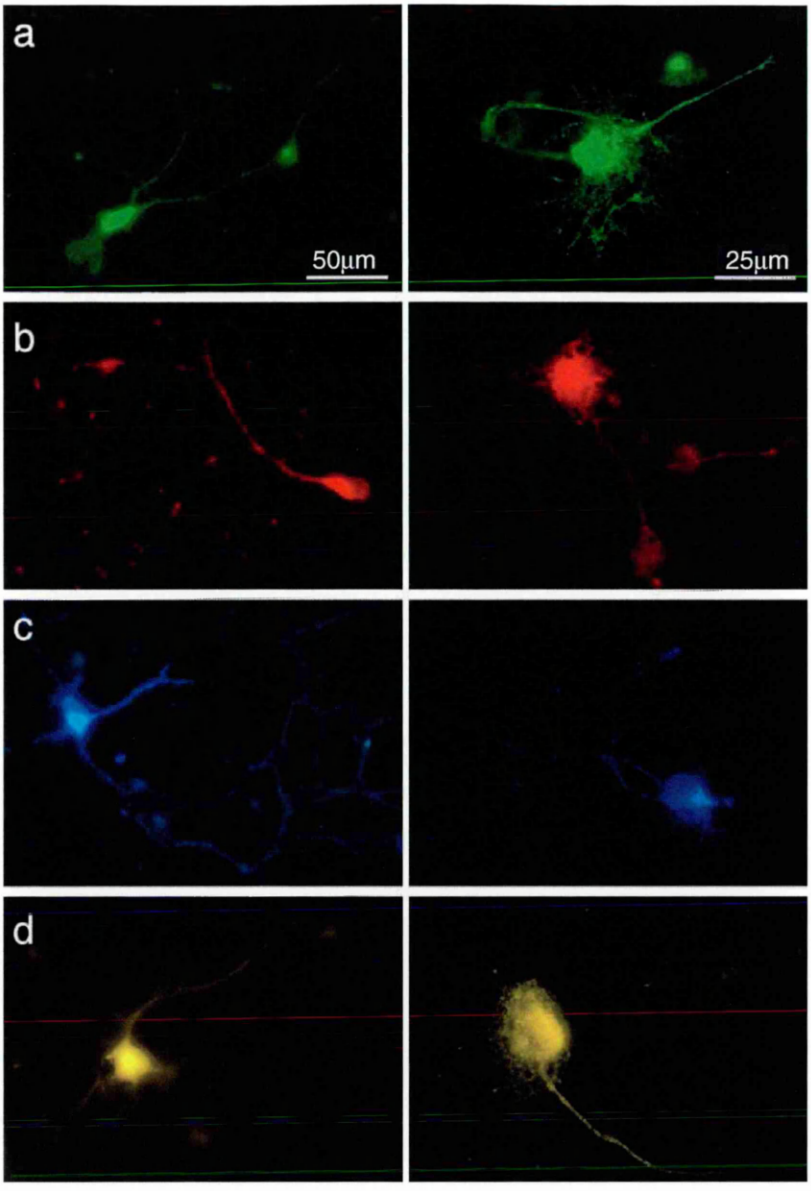


Figure 3. AMPA receptor subunit localization on motor neurons. Purified cultured motor neurons were stained with specific antibodies against GluR1 (a), GluR2 (b), GluR3 (c) and GluR4 (d) subunits.

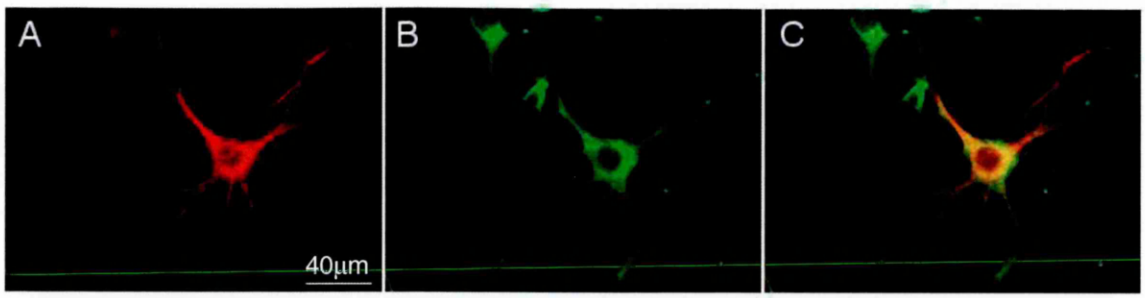


Figure 4. AMPA receptor subunit 2 is present on motor neuron.
Cocultured motor neurons were double-stained by SMI32 (A, red) and the specific antibody against GluR2 (B, green), without undergoing the cell permeabilization procedure. GluR2 is present both on motor neurons (merge in C) and on SMI32-negative cells (green in C).

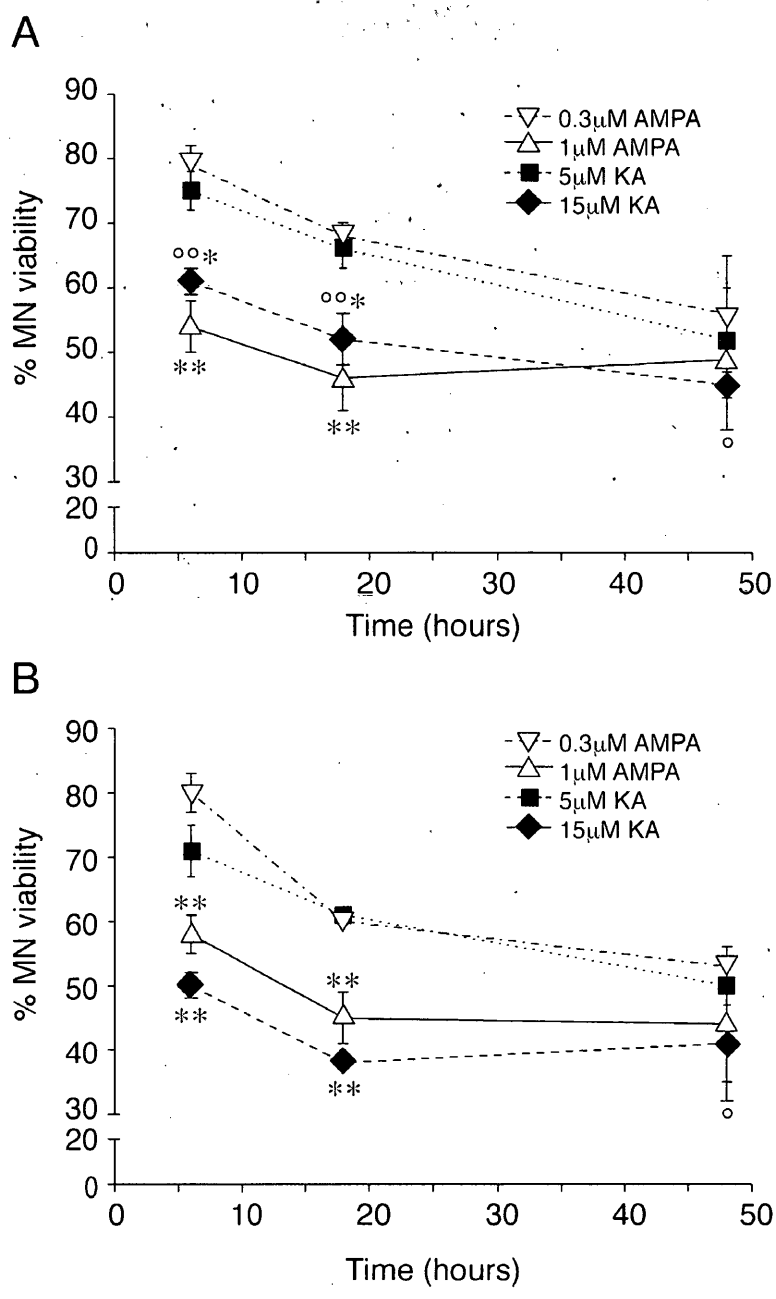


Figure 5. Time-course of different AMPAR agonist concentrations. Motor neuron viability after 6, 18 or 48h of treatment with different AMPAR agonist concentrations in mixed anterior horns cultures (panel A) or cocultures (panel B). Data represent means ± S.D. of SMI32-positive cells normalized for CTR. 6-9 wells for each condition have been analyzed. ** $p< 0.01$ vs 0.3 μM AMPA and 5 μM KA; * $p< 0.05$ vs 5 μM KA; °° $p< 0.01$ vs 0.3 μM AMPA; ° $p< 0.05$ vs 0.3 μM AMPA and 5 μM KA. One way ANOVA and Tukey test.

all in the axon hillock, but slight staining of neuronal arborizations was also evident.

We also detected the expression of GluR2 in SMI-32 positive motor neurons in cocultures which were not permeabilized by Triton (see Methods for details; Fig. 4). Our results were in agreement with previous findings on the expression of this AMPAR subunit on motor neurons purified by different purification procedures (Comoletti *et al.* 2001, Greig *et al.* 2000, Vandenberghe *et al.* 1998) and with evidence of functional GluR2-dependent properties (Van Damme *et al.* 2007, Van Damme *et al.* 2002).

The death kinetics mediated by the activation of AMPAR was evaluated by quantifying the motor neuron viability in mixed anterior horn cultures (Fig. 5A) or cocultures (Fig. 5B) after different times of exposure (6, 18 or 48 hours) to different concentrations of AMPA (0.3 or 1 μM) or kainate (5 or 15 μM). High AMPAR agonist concentrations (15 μM kainate or 1 μM AMPA) induced a relevant rate of death of SMI32-positive motor neurons shortly after treatment, reaching a "plateau" effect within 18 hours; while lower excitotoxic insults (0.3 μM AMPA or 5 μM kainate) lead to a significantly reduced cell death rate, progressively increasing to 50–60% death after 48 hours.

Both in mixed anterior horn cultures or in cocultures 15 μM kainate or 1 μM AMPA induced more than 35% of motor neuron death already after 6 hours of exposure, while lower concentrations triggered about 25% cell death ($p < 0.01$). A similar trend was shown after 18 hours, when higher AMPAR agonist concentrations induced between 50% (in mixed anterior horn cultures, Fig. 5A) and 60% (in cocultures, Fig. 5B) of motor neuron death, while lower concentrations failed to induce more than 40% toxicity ($p < 0.01$). After 48 hours of exposure, the effect of the different AMPAR agonist concentrations was quantitatively similar (about 50%). Only the effect of 15 μM kainate (55% of cell death) was statistically different ($p < 0.05$) from the effect of 5 μM kainate (50% death) or 0.3 μM AMPA (45% death).

Finally, we showed that co-treatment with the specific AMPAR antagonist NBQX (2 μM) significantly counteracted the motor neuron death induced both by low or high AMPAR agonist concentrations in cocultures (Fig. 6), suggesting that the neurotoxic effect was specifically mediated by the AMPAR, in accordance with the results obtained in a previous study in mixed anterior horn cultures (Comoletti *et al.* 2001).

6.2 Neurodegenerative pathways: caspase activation

For the detection of the caspase cascade activation, mixed anterior horn cultures or cocultures (representative picture in Fig. 8) were double stained using specific antibodies against SMI32 and the activated form of caspases-9 or -3. In control conditions, SMI32-positive motor neurons showed regular shape and size (Fig. 8, CTR, green cells) and slight activation of caspases-9 or -3 (quantified in Fig. 7A or B). After 6 hours, treatments with low kainate or AMPA concentrations reduced the number of SMI32-positive motor neurons and significantly increased the percentage of activated caspase-9- (Figs. 7A and 8) or -3- (Figs. 7B and 8) positive cells in both culture conditions.

1 μM AMPA or 15 μM kainate treatments induced a severe reduction in the viability of SMI32-positive cells and the remaining motor neurons showed a compromised morphology (Fig. 8, green cells). In cocultures (Fig. 7A or B, filled bars) the SMI32 positive cells showed a very low percentage of activated caspases-9 or -3 and in mixed anterior horn cultures (Fig. 7A or B, empty bars) not significant rate of caspase activation (probably due to the biological turnover of developing cells as well as to plating-dependent stress conditions) was observed after the high concentration treatment, suggesting that the caspase-dependent death pathway was not involved.

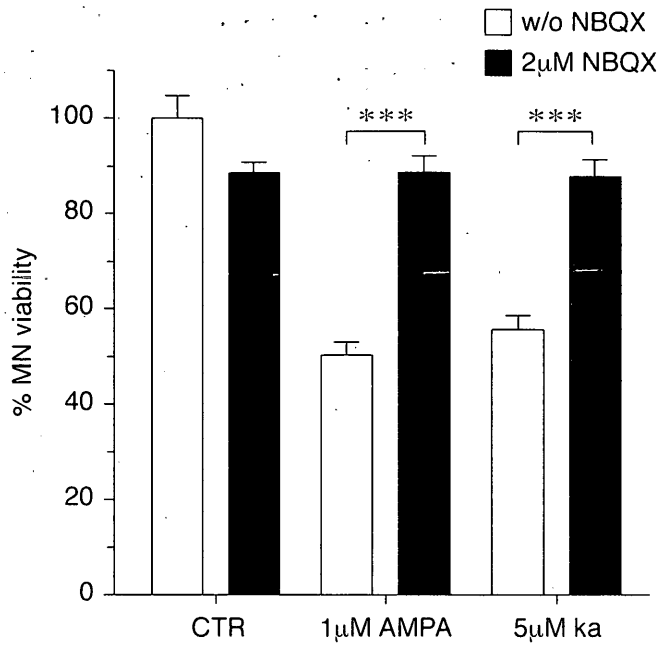


Figure 6. NBQX counteracts AMPAR agonist-mediated motor neuron death.

Motor neuron viability after 48h treatment of cocultures with low (5 μM kainate) or high (1 μM AMPA) AMPAR agonist concentrations, alone or in co-treatment with 2 μM NBQX. Data represent means ± S.D. of SMI32-positive cells normalized for CTR. 6 wells for each condition were analyzed. *** $p < 0.001$ vs treatment without NBQX. Two way Anova and Bonferroni post-test.

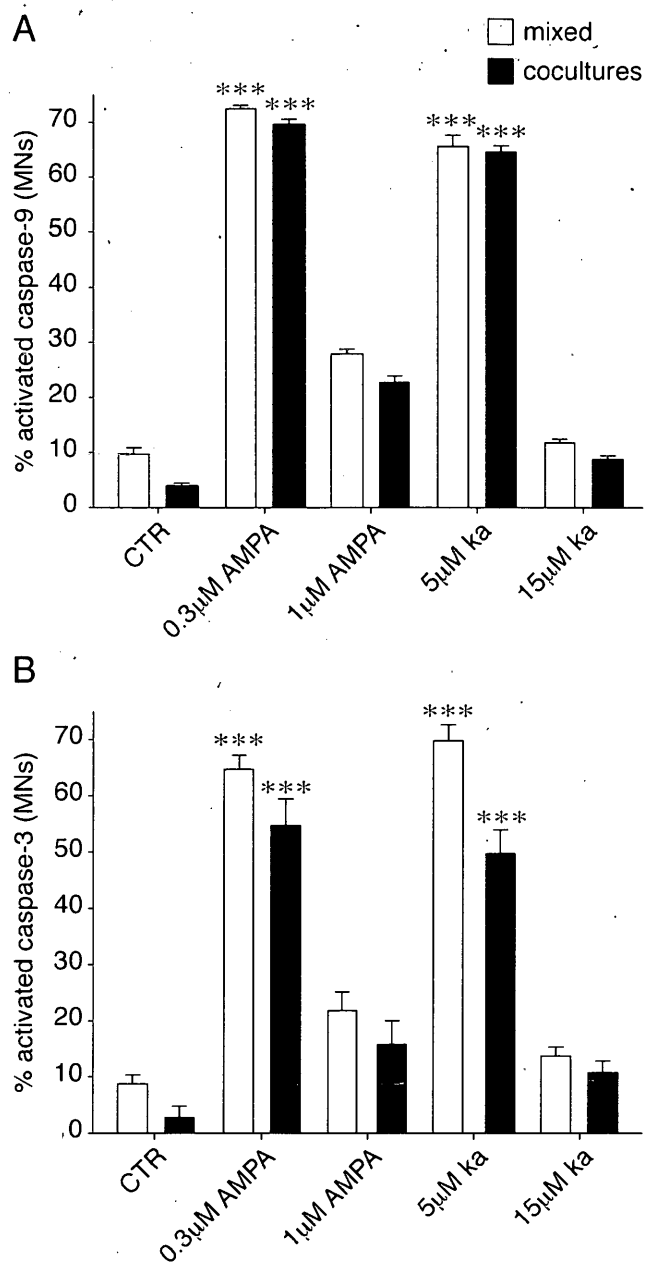


Figure 7. Activation of caspase-9 or -3 by AMPAR agonists.

Mixed anterior horn cultures or cocultures were exposed to different AMPAR agonist concentrations for 6 h and then double stained with SMI32 and specific antibodies against the activated form of caspases.

6 cover slips for each condition were analyzed. Only double-stained cells were counted. Bars represent mean percentage ± SD of motor neurons showing activation of caspase-9 (panel A) or caspase-3 (panel B) after each treatment in mixed anterior horn cultures (empty bars) or cocultures (filled bars). *** $p<0.001$ vs CTR, 0.3 μM AMPA and 5 μM KA. ANOVA and Tukey test.

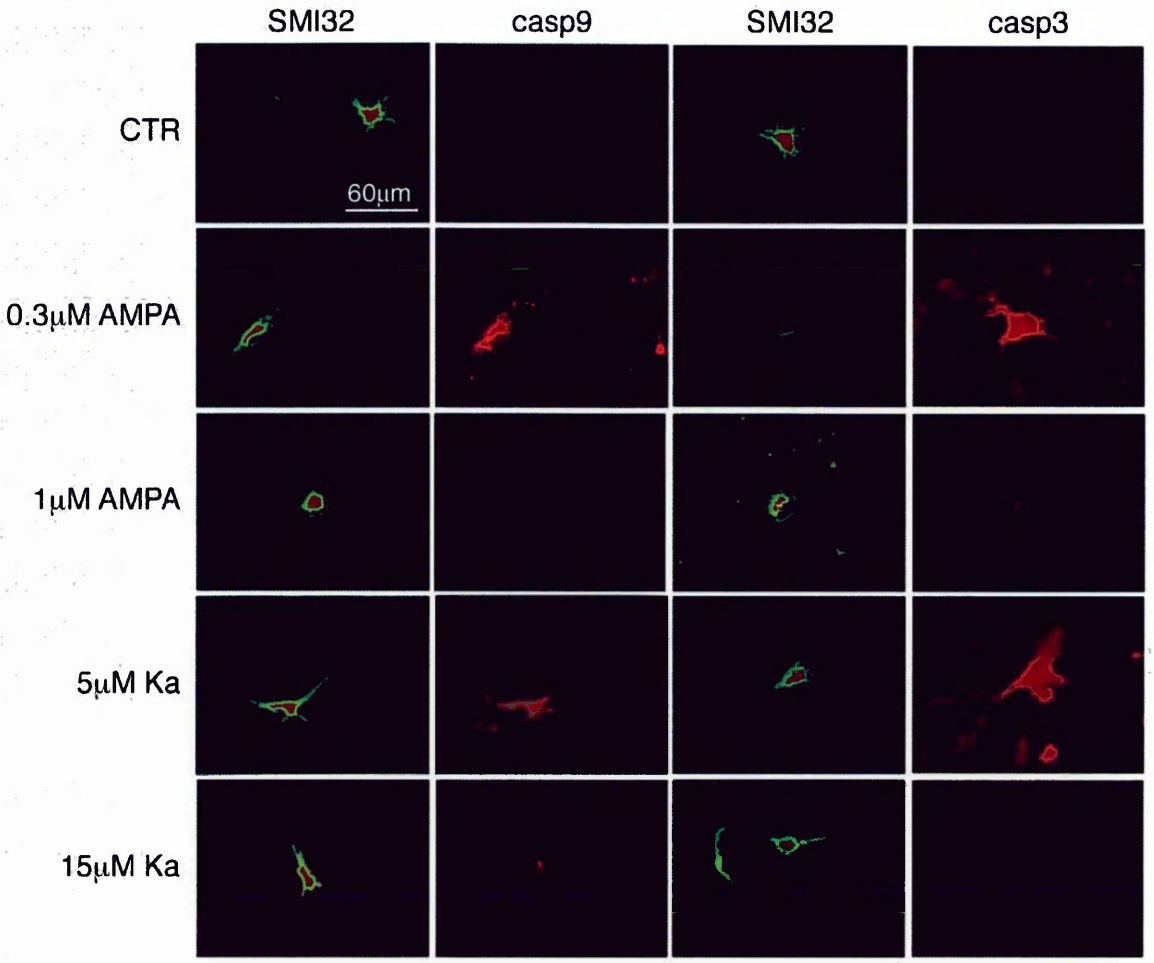
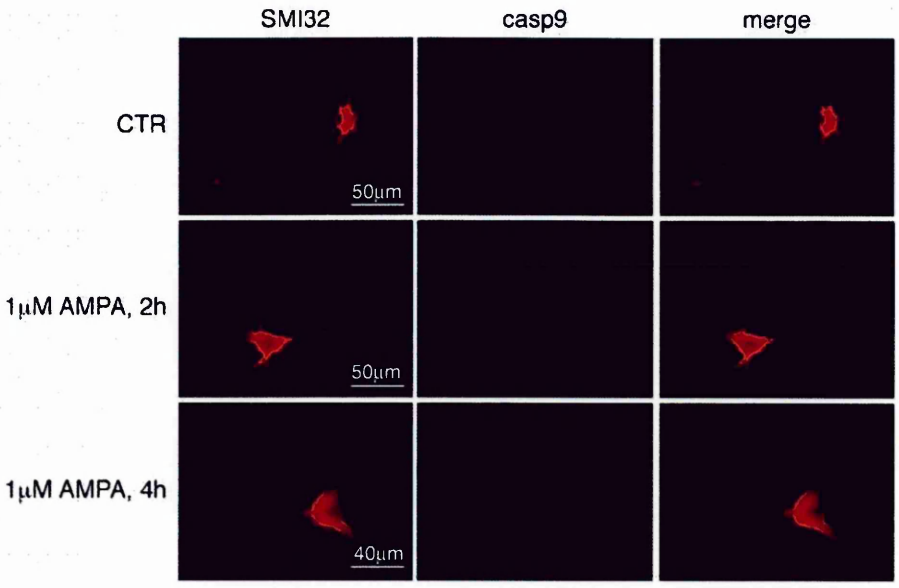


Figure 8. Activation of caspase-9 or -3 by AMPAR agonists in cocultures.

Representative pictures of motor neurons in cocultures exposed to different AMPAR agonist concentrations for 6 h and then double-stained by SMI32 (green) and specific antibodies against the activated form of caspase-9 or caspase-3 (red).

A



B

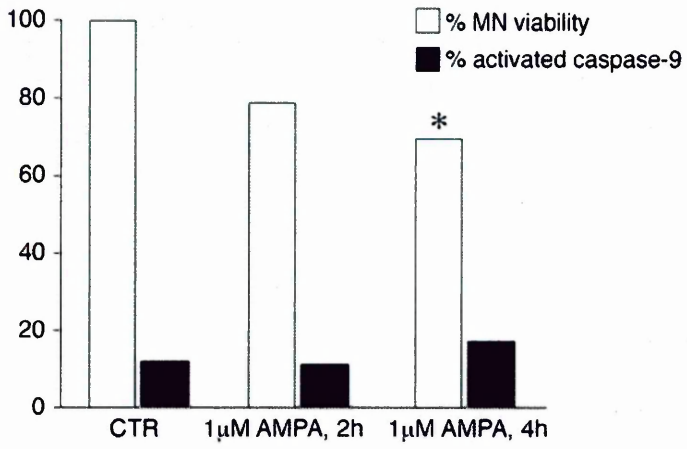


Figure 9. High AMPA concentration does not induce caspase-9 activation even at early times.

Cocultures were exposed to 1 μM AMPA for 2 or 4 h, then double-stained by SMI32 and a specific antibody against the activated form of caspase-9. Panel A: representative pictures of double stained SMI32 (red)/caspase-9 (green) motor neurons in cocultures. Panel B: 4 cover slips for each condition were analyzed. Graph bars represent the mean percentage ± SD of SMI32-positive motor neurons (empty bars) or the percentage of double stained (SMI32/caspase-9; filled bars) cells present in each treatment condition. * $p<0.05$ vs CTR, ANOVA and Tukey test.

To further verify that the lack of caspase activation after high AMPA concentration could not be related to a masking effect by the cells that have already died, we repeated the experiment in cocultures treated with 1μM AMPA at earlier times (2 or 4 hours). Motor neuron death was about 20% (after 2 h) or 35% (after 4 h) and no significant activation of caspase-9 was detected (Fig. 9B; representative pictures of stained motor neurons in Fig. 9A). Thus, since early times of exposure, high agonist concentrations did not induce caspase activation.

6.3 Neurodegenerative pathways: cytochrome *c* release

The key event in the intrinsic apoptotic pathway is represented by the release of cytochrome *c* from mitochondria into the cytosol (Neame *et al.* 1998). Cytochrome *c* is normally located in the space between the outer and the inner membrane of mitochondria. During apoptosis the pro-apoptotic molecules are activated and move into the mitochondria where they induce the release of cytochrome *c* (and other proteins) from the intermembrane space. Once diffused into the cytosol, cytochrome *c* is able to initiate the processing of multiple caspase activation.

In this study we verified whether treatment with AMPAR agonists induced this process. Purified motor neurons, were double stained with a mitochondrial dye (Mito Tracker) and an antibody directed against cytochrome *c*. Purified motor neuron cultures were treated with 50 μM kainate (representative of lower concentrations) or 10 μM AMPA (representative of the higher ones) for 4 hours; these concentrations were utilized since they exert an equipotent effect in inducing motor neuron death equal to 5 μM kainate or 1 μM AMPA in mixed anterior horn cultures after 48 hours of treatment (Comoletti *et al.* 2001).

In contrast to 10 μM AMPA treated motor neurons, in which a loss of mitochondrial integrity and a consequent spreading of cytochrome *c* immunoreactivity is already

detectable after 4 hours of treatment (Fig 10 C, F), both in control and in 50 μM kainate treated cells mitochondrial integrity was seen (Fig 10 A, B). However, in a large percentage (quantification not done) of kainate treated motor neurons, cytochrome *c* staining did not overlap with mitochondria, but homogenously diffused throughout the whole cytoplasm (Fig 10 E). This evidence supports the hypothesis that low AMPAR agonist concentrations can induce apoptosis by modifying mitochondrial activity and thus, allowing the caspase-cascade activation by cytochrome *c* release and consequent apoptosome formation.

6.4 Neurodegenerative pathways: phosphatidylserine externalization

We investigated also the effect of the different AMPAR agonist concentrations on a typical apoptotic alteration of the cell membrane such as the expression of phosphatidylserine residues on the membrane's outer leaflet (revealed by the annexin V binding). Phosphatidylserine is normally found on the cytosolic surface of the plasma membrane, but it is redistributed during apoptosis to the extracellular surface by a protein known as scramblase (Wang *et al.* 2003). This event marks the cell for the phagocytotic process, mediated by cells possessing the appropriate receptors, such as macrophages (Savill *et al.* 2003).

Cocultures were exposed to 6 hour treatment with the different agonists, then double-stained with SMI32 and annexin V antibodies. Semi-quantitative data, obtained by counting the double stained cells (SMI32/annexin V-positive cells; representative pictures in Fig. 11 A), showed that 5 μM kainate or 0.3 μM AMPA significantly increased the percentage of annexin V-positive motor neurons in cocultures, while higher concentrations did not induce this membrane alteration (Fig. 11B). After 0.3 μM AMPA or 5 μM KA treatment, also a high number of SMI32-negative cells were immunoreactive for annexin V (Fig. 11A, red cells in the merge column), suggesting

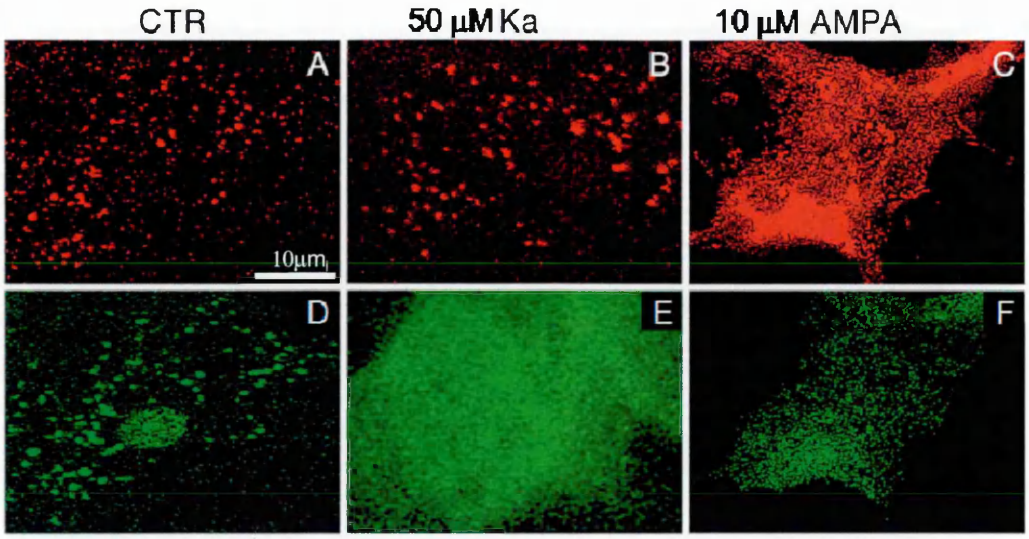


Figure 10. Cytochrome *c* release after treatment with low agonist concentration.

Representative pictures of purified motor neuron cultures double-stained with a mitochondrial dye (Mito Tracker, red signal) and an antibody directed against cytochrome *c* (green). After 6 h, 50 µM kainate treatment (B and E) induces a diffused release of cytochrome c from mitochondria, compared to control (A and D) or 10 µM AMPA-treated cells (C and F).

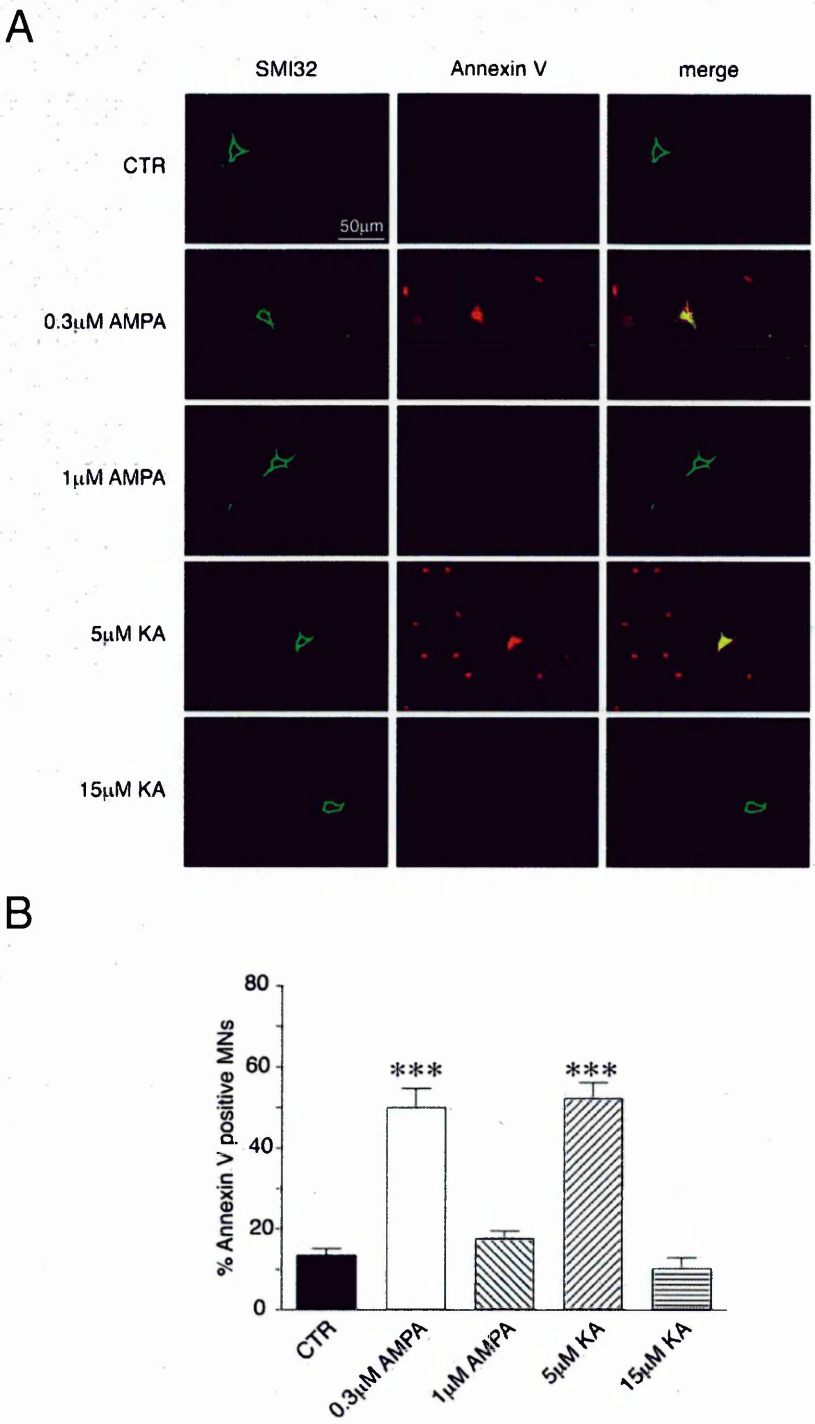


Figure 11. Low AMPA or kainate concentrations induce the externalization of phosphatidylserine residues on cell membranes. Cocultures were exposed to different AMPAR agonist concentrations for 6 hours and then double stained with SMI32 and the specific antibody for annexin V. Panel A: representative pictures of double stained SMI32 (green) / annexin V (red) motor neurons in cocultures. Both motor neurons (yellow in merge pictures) and SMI32-negative cells (red in merge pictures) showed positivity to the annexin V antibody after low concentration treatments. Panel B: 6 cover slips for each condition were analyzed. Bars represent the mean percentage ± SD of double-stained (SMI32/annexinV) cells. *** $p<0.001$ vs CTR or high concentrations, ANOVA and Tukey test.

that such apoptotic alteration did not occur only in motor neurons but rather it represents a common response of different cell types exposed to this treatment condition. These results confirmed that the phosphatidylserine membrane rearrangement was induced only by the lower agonist concentrations.

6.5 Neurodegenerative pathways: nuclear fragmentation

In 1990, Clarke described nuclear fragmentation as one of the main apoptotic alteration in cells: "In the nucleus, dense chromatin masses appear and increase in number until the nucleus has become frankly pyknotic (i.e., condensed). Under high magnification, the DNA fragments can be seen to be packed together more closely in the regions of dense chromatin." (Clarke 1990). DNA fragmentation was usually detected by TUNEL assay or DNA-binding dyes (i.e. Hoechst 33258) and reported as the main downstream event of the apoptotic process.

DNA fragmentation was studied here after 18 hours, when the effect of the low or high AMPAR agonist concentrations on motor neuron survival was significantly different (about 35% vs. 50% death, $p < 0.01$). In mixed cultures a relevant rate of DNA fragmentation was detected also in control conditions (about 20%) and was maintained after the exposure to high agonist concentrations (Fig. 12A). On the contrary, motor neurons in cocultures showed a very low amount of fragmented nuclei in controls and no DNA fragmentation after treatment with high agonist concentrations (Fig. 12B). After 18 hour treatment, the double staining of SMI32 and Hoechst 33258 revealed that 5 μM kainate or 0.3 μM AMPA significantly increased the nuclear fragmentation of SMI32-positive motor neurons compared to both control condition, 15 μM kainate or 1 μM AMPA treatment, both in mixed anterior horn cultures (Fig. 12A) and in cocultures (Fig. 12B; representative pictures of fragmented nuclei after low concentration treatments in Fig. 13). Again, lower concentrations, but not the higher, clearly induced

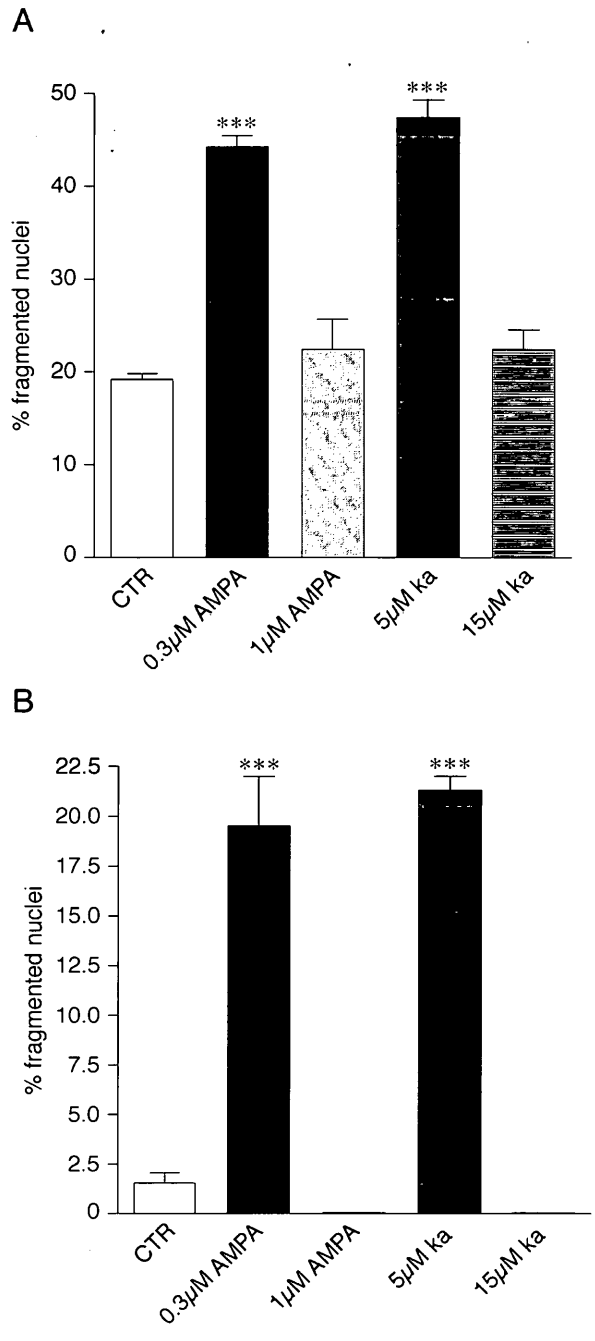


Figure 12. DNA fragmentation induced by different AMPAR agonist concentrations in mixed anterior horn cultures or in cocultures.
Mixed anterior horn cultures (A) or cocultures (B) were exposed to 5 or 15 μM kainate, 0.3 or 1 μM AMPA for 18 h. Cells were double-stained with SMI32 and Hoechst 33258. 8-10 cover slips for each culture condition were analyzed at a magnification of 200X. Only SMI32-positive cells were considered and their nuclei were analyzed at a higher magnification (600X). Nuclei that clearly showed fragmentation were counted. Graph bars represent the mean percentage ± SD of fragmented nuclei of SMI32 positive cells in each treatment condition. *** $p < 0.001$ vs CTR, 15μM KA or 1μM AMPA. One way ANOVA and Tukey test.

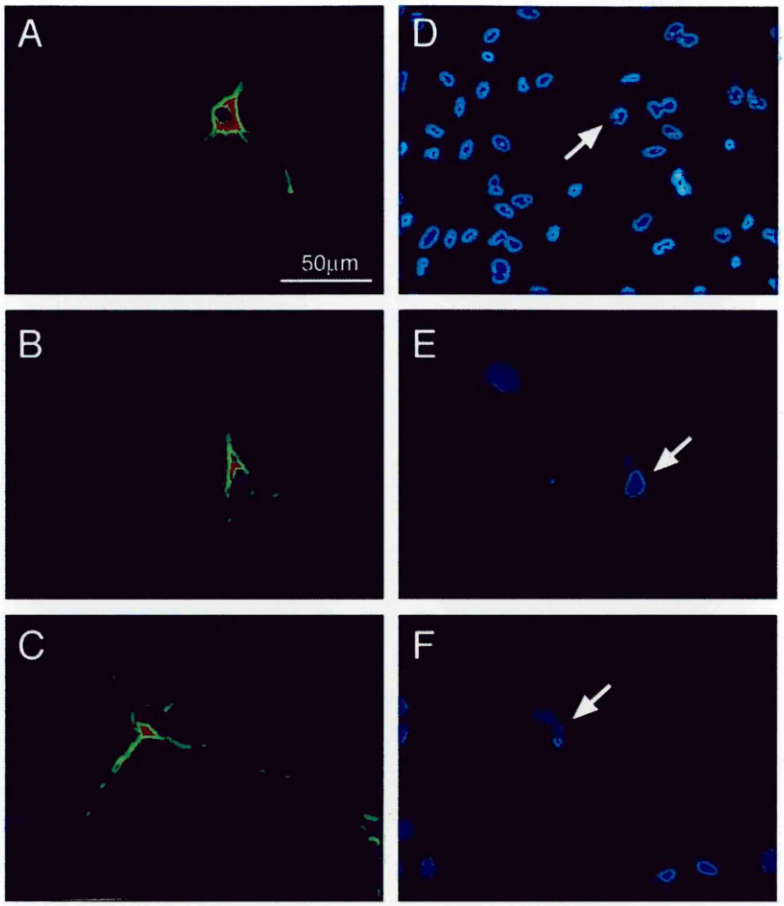


Figure 13. Low AMPAR agonist concentrations induce nuclear fragmentation in SMI32-positive motor neurons.

Cocultures, treated with 0.3 µM AMPA or 5 µM kainate for 18 h, were double-stained by SMI32 (A, B, C) and Hoechst 33258 (D, E, F). Arrows in pictures E and F point representative fragmented nuclei after treatments with respectively 0.3 µM AMPA or 5 µM kainate. Nuclei of control motor neurons appear intact and round-shaped (arrow in picture D).

classical apoptotic alterations, independently from the type of agonist (kainate or AMPA).

6.6 AMPAR activation induced cytosolic calcium alterations

To investigate possible alterations in AMPAR-dependent calcium influx we evaluated the intracellular Ca^{2+} concentrations of motor neurons exposed to different AMPAR agonists by two different techniques. In the first one, we tried to identify possible differences in cytosolic or mitochondrial Ca^{2+} influx in motor neurons exposed to higher or lower kainate concentrations. Cocultures were loaded with specific indicators for cytosolic and nuclear (calcium green) or mitochondrial (RHODD1) calcium. A confocal microscope was used to reveal alterations in calcium-dependent fluorescence in motor neurons (identified in cocultures by morphological criteria). Treatment with AMPAR agonists was performed by superfusing the solutions in a watertight chamber. Pictures were obtained at different times after perfusion with the agonists and maintaining the microscope acquisition setting steady. We found that low AMPAR agonist concentrations induced increases in cytosolic calcium levels. Figure 14 shows pictures acquired under basal conditions and 3 min after 5 μM kainate exposure. By subtracting the fluorescence of the basal picture from that acquired after 3 min kainate exposure, we revealed that calcium-dependent fluorescence was clearly increased by the excitotoxin both in cells showing glial phenotypes as well as in neuronal arborizations, and it was likely localized in the membrane compartment (Fig 14; examples of cells showing neuronal phenotype in the magnifications). Moreover, when we did the subtraction of the picture of treated cells from the basal fluorescence, we obtained a complete black field (Fig 14), thus confirming that calcium-dependent fluorescence was increased by kainate treatment. On the other hand, a higher kainate concentration (15 μM) appeared to induce a redistribution of intracellular Ca^{2+} rather than a net increase in $[Ca^{2+}]_i$.

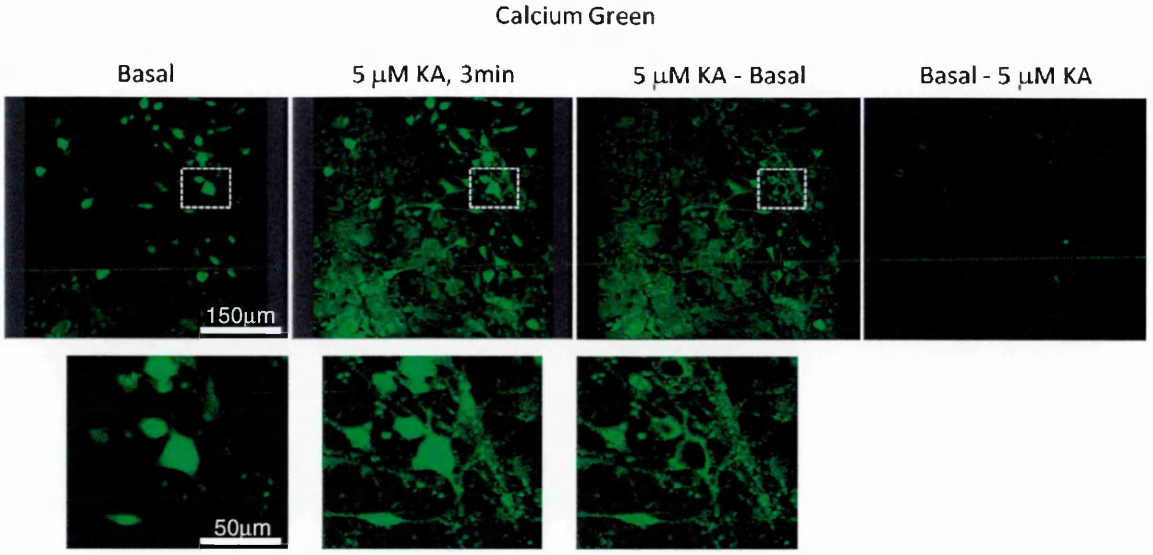


Figure 14. Effect of low AMPAR agonist concentration on cytosolic calcium influx (confocal microscopy).

Cocultures were loaded with specific indicators for cytosolic and nuclear calcium (calcium green). Alterations of calcium-dependent fluorescence in motor neurons (identified in cocultures by morphological criteria) were revealed by confocal microscopy after the perfusion of 5 μM kainate in a watertight chamber. Higher magnification of cells displaying neuronal-like phenotype is shown in the lower row.

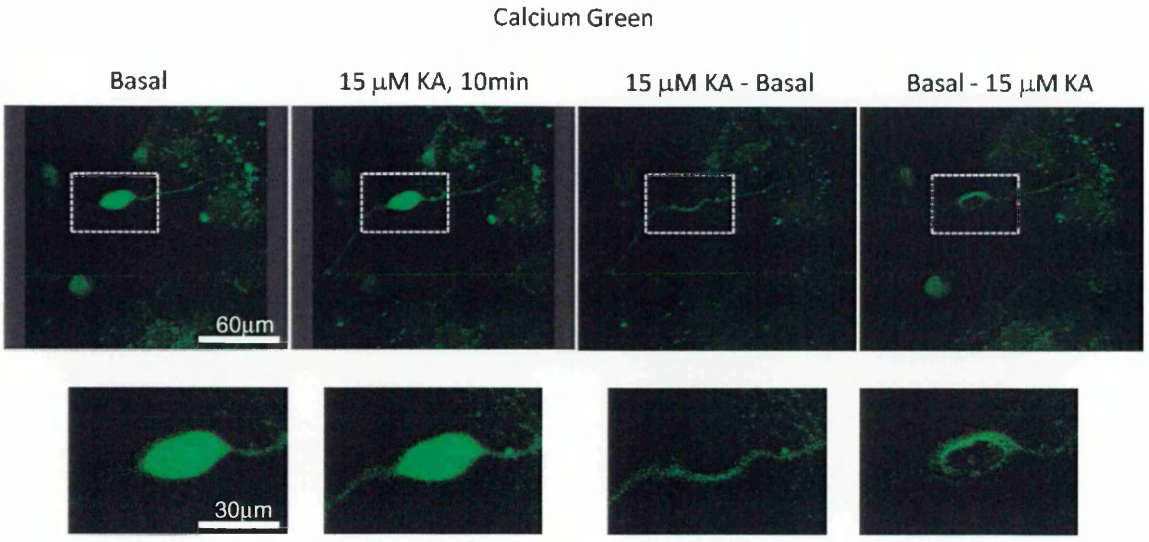


Figure 15. Redistribution of cytosolic calcium by treatment with high AMPAR agonist concentration (confocal microscopy).

Cocultures were loaded with specific indicators for cytosolic and nuclear calcium (calcium green). Alterations in calcium-dependent fluorescence in motor neurons (identified in cocultures by morphological criteria) were revealed by confocal microscopy after the perfusion of 15 μM kainate in a watertight chamber. Higher magnification (800X) of cells displaying neuronal-like phenotype is shown in the lower row.

Since a slight increase in CG5AM fluorescence after 10 min of 15 μM kainate treatment was revealed when the fluorescence of basal picture was subtracted from that acquired after kainate treatment or when we did the inverse subtraction of pictures (basal – kainate treatment), this would suggest that Ca^{2+} ions were re-localized with a different intracellular distribution after 15 μM kainate exposure (Fig 15). With the calcium indicator selectively sequestrated by mitochondria we also revealed a trend to an increase in mitochondrial calcium levels, in particular after treatment with low agonist concentrations (5 μM kainate, not shown).

The features of the confocal microscopy technique, while providing the accuracy of confocal microscope analysis, introduced a series of difficulties, the most important of which concerned time-lapse detection and quantification of the calcium influx. For these reasons we used a more suitable method for live imaging based on the epifluorescent microscopy (detailed description in section 4.4). Briefly, cocultures were loaded with Fura 2 AM fluorescent dye which allowed the expression of a fluorescent signal as the ratio between fluorescence emission at 340 and that at 380 nm. Increasing free (not bound, emission at 340 nm) Ca^{2+} concentrations led to an increased 340/380 fluorescence ratio. Here we detected the $[Ca^{2+}]_i$ variations after treatment with the AMPAR agonist concentrations. Traces of $[Ca^{2+}]_i$ kinetics were obtained from the time-lapse recordings of fields containing at least one morphologically identified motor neuron and some cells displaying the neuronal phenotype.

A first set of experiments were performed to evaluate and standardize the sensitivity of the method. In particular, we verified that motor neurons in cocultures showed detectable $[Ca^{2+}]_i$ variations after membrane depolarization induced by 50mM KCl and tested the sensitivity of the Fura 2 AM dye in the presence of physiological (1.8 mM) or saturating (10 mM) extracellular $[Ca^{2+}]$ (not shown). When the effect of the AMPAR agonists was studied, we could reveal rapid $[Ca^{2+}]_i$ variations after the agonist exposure

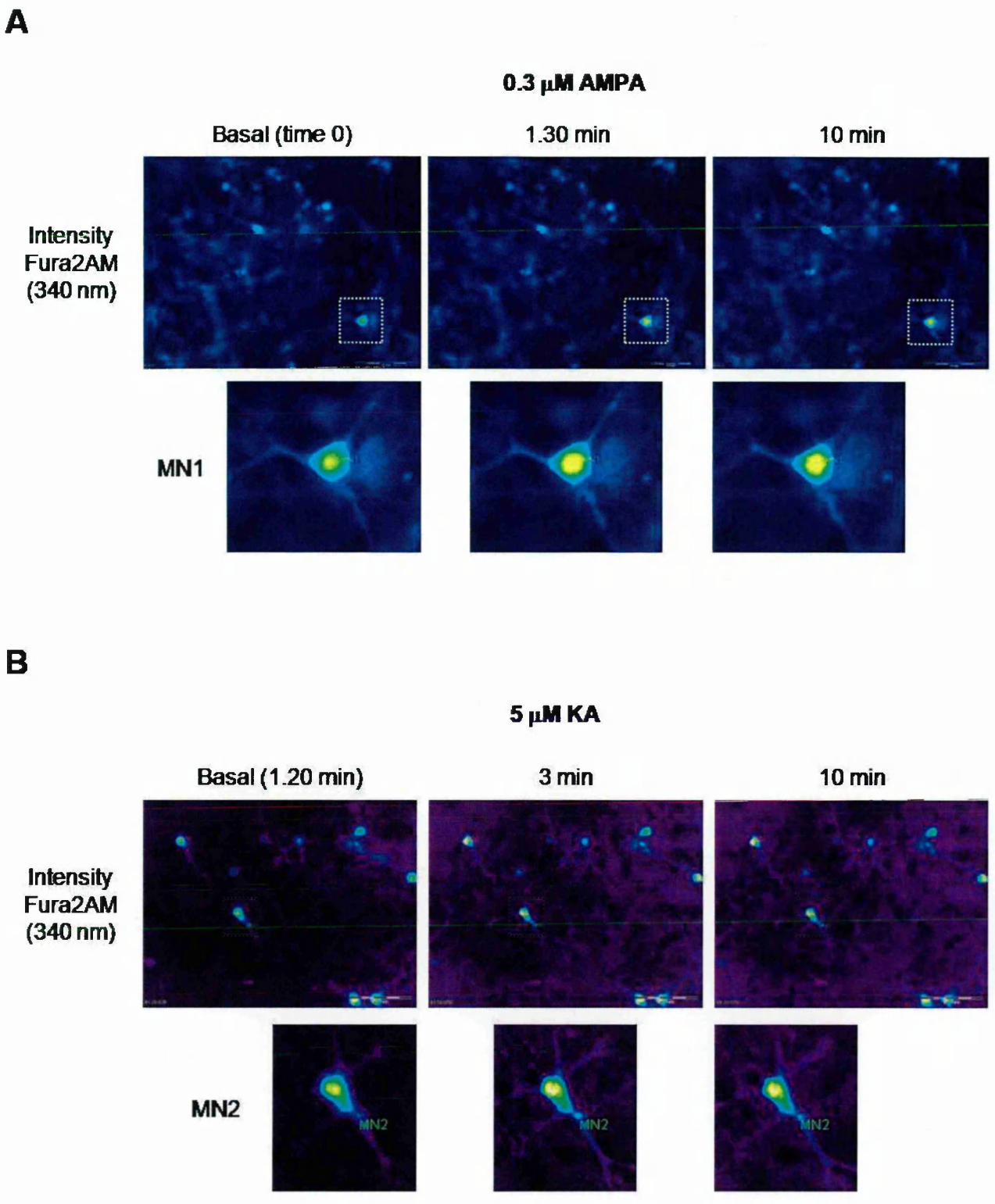

A
0.3 µM AMPA
Basal (time 0)
1.30 min
10 min
Intensity
Fura2AM
(340 nm)
MN1
B
5 µM KA
Basal (1.20 min)
3 min
10 min
Intensity
Fura2AM
(340 nm)
MN2
MN2
MN2
MN2

Figure 16.

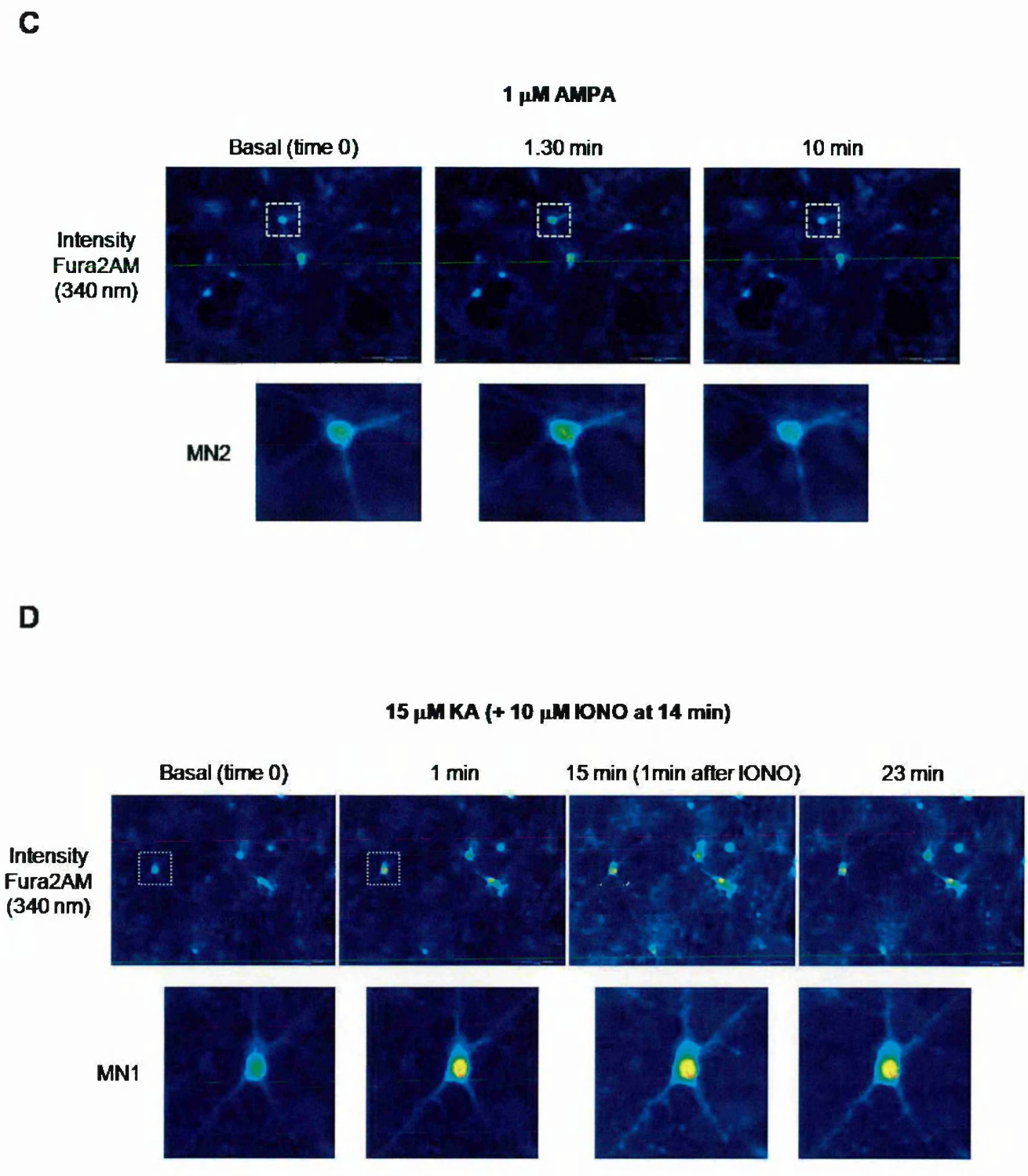


Figure 16. AMPAR agonists evoke $[Ca^{2+}]_i$ increase in cocultured motor neurons (live imaging).

Cocultures were loaded with Fura-2 AM and basal $[Ca^{2+}]_i$ levels were recorded before addition of AMPAR agonists. Here fluorescent images taken at 340 nm in basal condition or at different times from 0.3 (A) or 1 µM (C) AMPA or 5 (B) or 15 (D) µM kainate addition are reported.

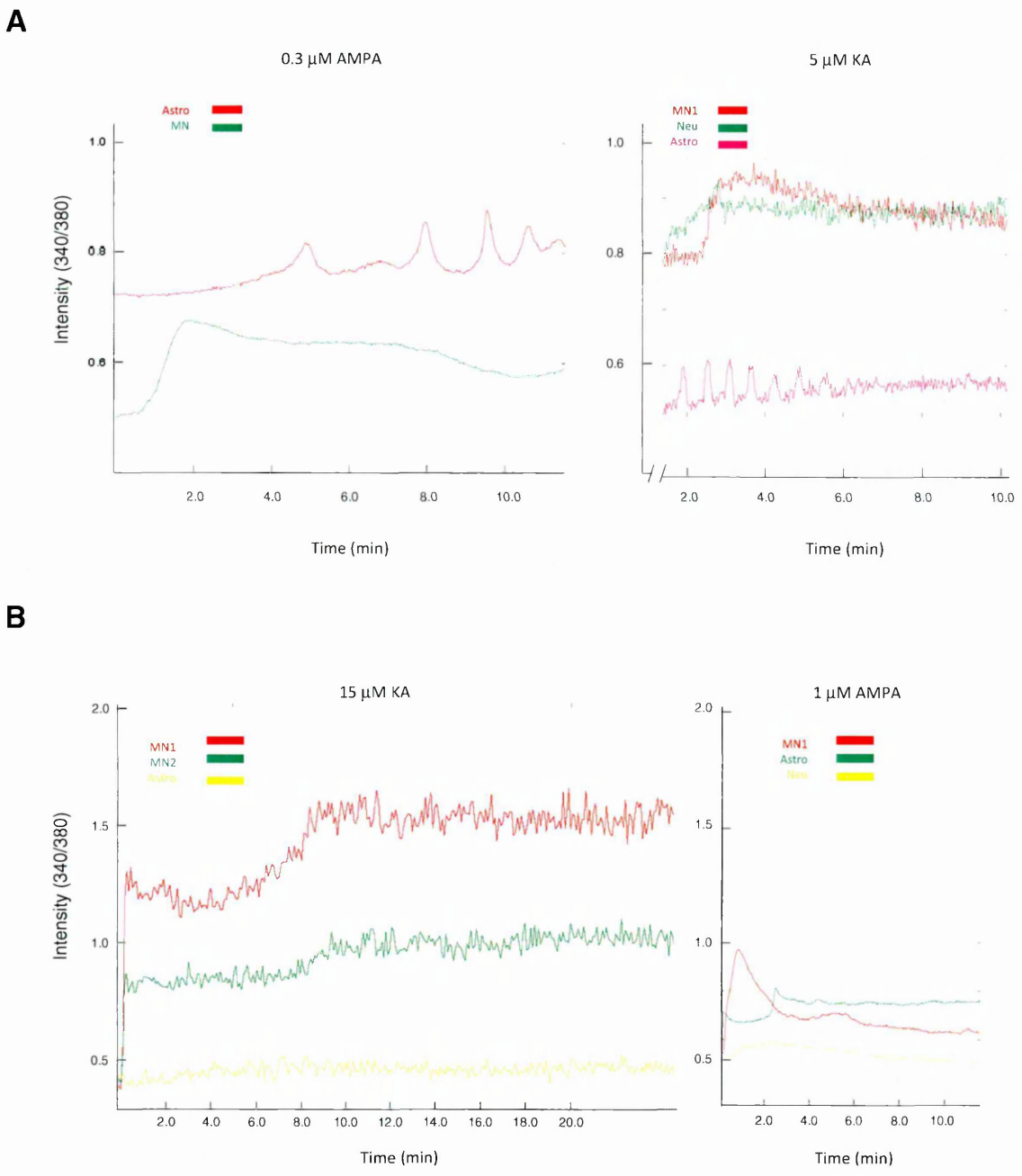


Figure 17. $[Ca^{2+}]_i$ rise induced by AMPAR agonist addition.

Cocultures were loaded with Fura-2 AM and time-lapse recording of $[Ca^{2+}]_i$ were obtained with a live-imaging equipment. Traces represent the kinetics of $[Ca^{2+}]_i$ alterations in cells displaying motor neuronal, neuronal or astrocytic phenotypes (defined by morphological criteria) after low (A) or high (B) AMPAR agonist concentration addition to the medium. High agonist concentrations induce higher increase of $[Ca^{2+}]_i$ as the increase of intensity R340/380 is greater after 15 µM KA or 1 µM AMPA, compared to that induced by the lower concentrations.

and for all the agonist concentrations tested (Fig 16 and 17). After exposure to high AMPAR agonist concentrations a higher variation in $[Ca^{2+}]_i$ was detected, compared to that revealed after treatment with the lower ones. In fact 1 μM AMPA (Fig 17B, representative pictures obtained from the time-lapse recording in Fig 16C) or 15 μM kainate (Fig 17B, representative pictures in Fig 16D) induced a higher (about 0.5) increase in intensity $R_{340/380}$ compared to 0.3 μM AMPA treatment (Fig 17A, representative pictures in Fig 16A) or 5 μM kainate (Fig 17A, representative pictures in Fig 16B) which triggered about 0.1-0.2 increase in intensity $R_{340/380}$. To demonstrate that the revealed increase in $[Ca^{2+}]_i$ was dependent on AMPAR activation, we verified that the calcium influx induced by the AMPAR agonists was counteracted by concomitant exposure to 2 μM NBQX (AMPAR antagonist, not shown). Interestingly, by this method we were also able to detect a rhythmical oscillation of the calcium concentration in astrocytes (evident in the 5 μM kainate treatment, trace from astrocyte in pink, Fig 17A) that did not seem to be affected by the excitotoxic treatment.

Conclusions

Our data demonstrated that, in spite of a better phenotype of motor neurons in cocultures, there are no differences from the mixed anterior horn cultures in terms of sensitivity to the AMPAR-mediated neurotoxicity, excluding a possible toxic effect of OptiPrep on purified motor neurons. In fact, independently from the type of agonist (AMPA or kainate), higher concentrations induced relevant death rates after 6 hours, while lower concentrations induced a milder, progressively increasing effect showing similar concentration-dependent death curves in both culture conditions. To detect the effect of the different agonist concentrations on the intracellular mechanisms leading to motor neuron death, we considered early events of the apoptotic pathway (such as the

activation of the caspase cascade and the membrane externalization of annexin V) and a later event such as the DNA fragmentation. Since the death curves of the AMPAR agonist concentrations showed the higher statistically significant differences after 6 or 18 hours exposure, we considered these as time points with the better chance of detecting possible differences in inducing intracellular death pathways. High concentrations of AMPAR agonists inducing more than 35% of motor neuron death within 6 hours did not appear to activate the apoptotic mechanisms, while lower concentrations, which induced less than 25% death, led to the activation of typical apoptotic intracellular markers such as the cleavage of caspases-9 and -3, the expression of phosphatidylserine residues on the membrane's outer leaflet and the DNA fragmentation. The lack of activation of the apoptotic death pathway by high AMPA concentration was not related to the high death rate, since no activation of caspase-9 was found even 2 or 4 hours after treatment with 1 μM AMPA, when motor neuron death was only about 20%, quantitatively similar to that obtained after 6 hours of treatment with the lower concentration, when caspase activation was shown. Thus, our data demonstrated that the activation of the AMPAR induced by different excitotoxic stimuli triggered specific intracellular death mechanisms, which are related neither to the morphological differences found in motor neurons cultured with immature glial cells (mixed anterior horn cultures) as compared to those with glial cells grown up to 4 weeks in vitro (cocultures), nor to the type of agonist but are rather dependent on the intensity of the initial stimulus.

Our results, demonstrating that the two AMPAR agonist concentrations tested here were able to trigger rapid $[Ca^{2+}]_i$ increase of different amplitude, led to the hypothesis that the higher $[Ca^{2+}]_i$ variation induced by the high AMPAR agonist concentrations (1 μM AMPA or 15 μM kainate) was the triggering event leading to the rapid, non-apoptotic motor neuron death shown in this toxic condition (see section 6.1). On the other hand,

low AMPAR agonist concentrations induced a lower $[Ca^{2+}]_i$ variation, which could allow the motor neuron to recover from this perturbation or to start the programmed cell death mechanism in case of a persisting unbalanced situation. This finding fits very well with the evidence reported in literature supporting the fact that sustained or excessive AMPAR-mediated Ca^{2+} influx, together with excessive mitochondrial Ca^{2+} loading (Sen *et al.* 2008) and lower buffering capacity (Alexianu *et al.* 1994, Reiner *et al.* 1995) of motor neurons are the key factors which could account for their selective vulnerability to excitotoxicity.

Finally, although we did not examine this event carefully, the repeated low-amplitude calcium-dependent spikes shown by astrocytes in cocultures could be bona fide considered, in accordance with the literature (Parri & Crunelli 2001, Parri & Crunelli 2003, Parri *et al.* 2001), as spontaneous $[Ca^{2+}]_i$ oscillations which account for the intrinsic astrocytic activity also taking place in control conditions and independently from the neuronal activity or excitotoxic stimuli.

Chapter 7 - Neuroinflammation and excitotoxicity

7.1 Tumor Necrosis Factor-α

Background

The inflammatory response occurs in several brain trauma and neurological diseases. Cells that more actively participate to this process in the CNS are microglial cells and astrocytes, for this reason this response is often indicated as reactive gliosis (Raivich *et al.* 1999). Microglial cells are functionally related to peripheral tissue macrophages and, like macrophages in other tissues, resting microglia appears to participate in the immune surveillance of the nervous system (Eglitis *et al.* 1987). In response to injury, microglial cells modify their physiology and rapidly transform from a resting into an activated state. This is the triggering event of a series of cell alterations making microglial cells able to remove neural debris through phagocytosis.

The cellular modifications occurring in reactive astrocytes also play an important role in neuroinflammation. The morphological transformation from protoplasmic to fibrillary astrocytes in the neural parenchyma can be tightly controlled by several factors, including cytokines, which regulate different steps in this activation response (Lucas & Hohlfeld 1995). The release of cytokines can activate cellular pathways leading to the death of motor neurons. In addition diffusible factors released from dying neurons can, in turn, activate resting astrocytes (Viviani *et al.* 2000). Recent findings suggest that glial cells (astrocytes and microglia) play a role in motor neuron degeneration (Barbeito *et al.* 2004, Rao & Weiss 2004, Sargsyan *et al.* 2005). Although contrasting results have been reported for cytokine levels in the cerebrospinal fluid or plasma of ALS patients, high concentrations of IL-6, TNF and MCP-1 suggest a neuroinflammatory component (Baron *et al.* 2005, Cereda *et al.* 2008, Ford & Rowe 2004, Gallo *et al.* 1994, Joerg Stuerenburg *et al.* 1999, Krieger *et al.* 1992, Moreau *et al.* 2005, Poloni *et al.* 2000, Sekizawa *et al.* 1998, Wilms *et al.* 2001). Inflammatory reactions have been implicated

in several pathogenic mechanisms of motor neuron diseases (Appel *et al.* 1995, Cereda *et al.* 2008, Poloni *et al.* 2000) and the TNF signalling pathway has been demonstrated to mediate both apoptotic and necrotic cell death (Wallach *et al.* 1999). Since TNF-α has been shown to strengthen the glutamate-mediated neurotoxicity in human foetal neuronal cultures (Chao & Hu 1994) and injection of kainic acid increases the level of TNF-α mRNA in rat brain (Minami *et al.* 1991), the interactions between TNF signalling and excitotoxic injuries could represent a relevant contribution to motor neuron degeneration (Ghezzi & Mennini 2001).

Starting from these data, the present studies were aimed at investigating the effect mediated by exogenous TNF-α on motor neurons and its interaction with AMPA-mediated motor neuron degeneration. The contribution of glia to such an effect was elucidated by comparing motor neuron viability in different culture conditions and by detecting the TNF receptor localization in cocultures.

7.1.1 Effect of TNF-α treatment on motor neuron cultures

In a first set of experiments, mixed anterior horn cultures were treated with different concentrations of TNF-α alone or in co-treatment with 1 μM or 0.3 μM AMPA for 48 hours. TNF-α (tested at 1, 10 or 100 ng/mL) did not induce any toxic effect on SMI32-positive motor neurons (Fig 18) and did not affect the rate of motor neuron death induced by AMPA concentrations (Fig 19). Pre-treatment with the cytokine was also performed in order to verify whether a longer period of incubation was required. Cultures were pre-treated with 10 or 100 ng/mL TNF-α for 72 hours before 0.3 μM AMPA was added and treatment extended for a further 48 hours. Also with this protocol TNF-α showed no effect on motor neuron viability (not shown).

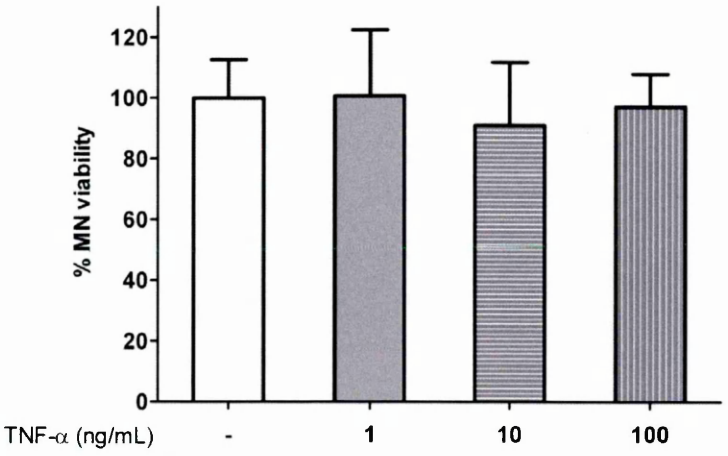


Figure 18. TNF-α treatment does not affect motor neuron viability in mixed anterior horn cultures.

Mixed anterior horn cultures were treated with different concentrations of TNF-α and then stained with SMI32. 6 cover slips for each condition were analyzed. Bars represent the mean percentage ± SD of SMI 32-positive cells. Treatments do not induce significant effect at any tested concentration.

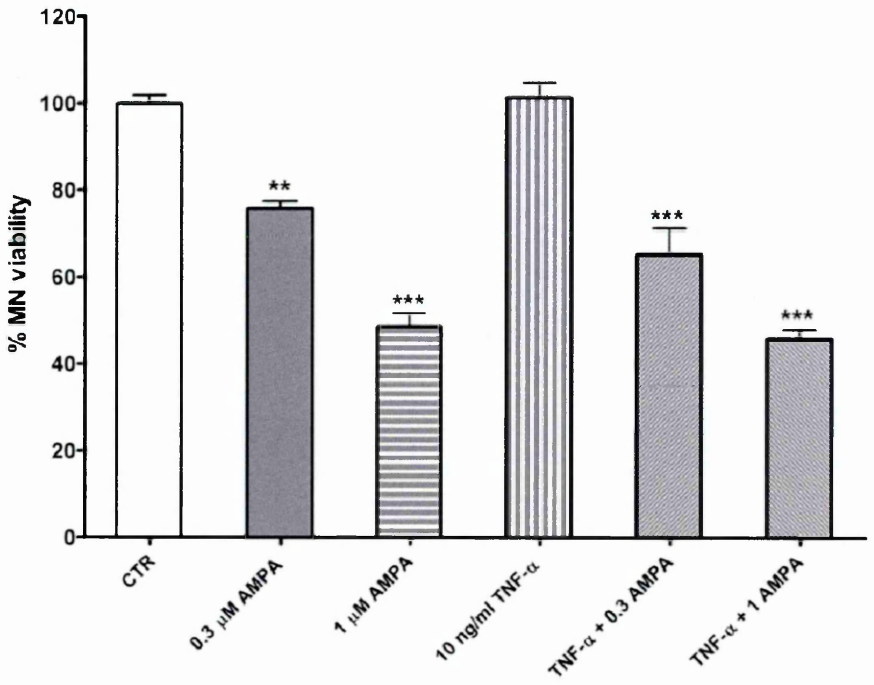


Figure 19. TNF-α treatment does not affect the neurotoxic effect of AMPAR agonists in mixed anterior horn cultures.

Mixed anterior horn cultures were treated with 10 ng/mL TNF-α alone or in co-treatment with 0.3 or 1 μM AMPA for 48 h. Bars represent the mean percentage ± SD of SMI 32-positive cells in each treatment condition. TNF-α treatment is not effective at any tested condition .

*** $p < 0.001$, ** $p < 0.01$ vs CTR or 10 ng/mL TNF-α. ANOVA and Tukey test.

We then tested 10 ng/mL TNF-α and its interactions with AMPAR agonist concentrations in cocultures to verify whether the confluent and mature glial layer would affect the cytokine effect. When cocultures were exposed to the cytokine for 48 hours, a significant motor neuron death (29.2 ± 8 %, $p < 0.01$ vs. control; Fig 20A) was observed. The addition of 0.3 or 1 μM AMPA induced about 35% or 47% of motor neuron death respectively when used alone, and these effects were significantly attenuated (by 16 or 15% respectively) by TNF-α co-treatment (Fig 20A) indicating an interaction between the two compounds. Indeed the toxic effect of TNF was no more evident in the presence of AMPA concentrations (Fig 20A) and the presence of TNF resulted in protection against AMPA-induced neurotoxicity.

Similar results were obtained if cocultures were pre-treated with 10 ng/mL TNF-α for 72 hours before the 48 hour incubation. In this case the cytokine toxicity was enhanced to about 50% of motor neuron death when used alone (Fig 20B). A significant interaction between TNF-α and AMPA was detected ($p < 0.001$, Two Ways Anova), since the co-treatment with cytokine decreased the toxicity of 0.3 or 1 μM AMPA from 38% or 52% (when used alone) to 22% or 38% (if in co-treatment with 10 ng/mL TNF-α) of motor neuron death respectively (Fig 20B).

Further studies on the specific contribution of glial cells to TNF-α-mediated toxicity were done by conditioning the confluent glial layer with exposure to the cytokine for 72 hours before seeding motor neurons and establishing the cocultures. The number of motor neurons grown in cocultures with conditioned glia was significantly lower (37% less, $p<0.001$; Fig 21) than that of motor neurons on control (not treated) glia.

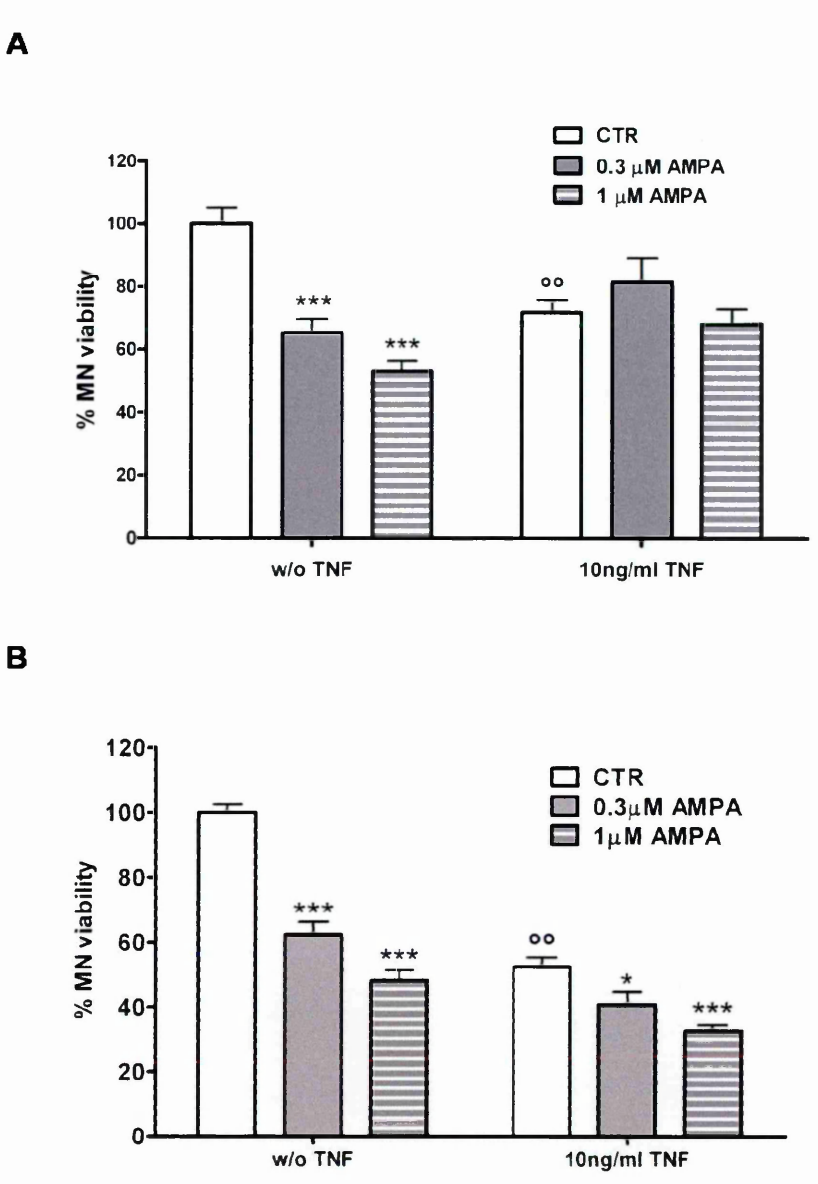


Figure 20. TNF-α induces motor neuron death in cocultures and significantly interacts with AMPAR agonist treatment.

Panel A: cocultures were treated with 10 ng/mL TNF-α alone or in co-treatment with 0.3 or 1 μM AMPA for 48 h. Panel B: cocultures were pre-treated with 10 ng/mL TNF-α (where present) for 72 h and then incubated with AMPA (in co-treatment with the cytokine where indicated) for further 48 h. Bars represent the mean percentage ± SD of SMI 32-positive cells in each treatment condition. *** $p < 0.001$, ** $p < 0.01$ vs CTR or 10 ng/mL TNF-α. Two way ANOVA and Bonferroni post tests, F_{int} $p < 0.001$.

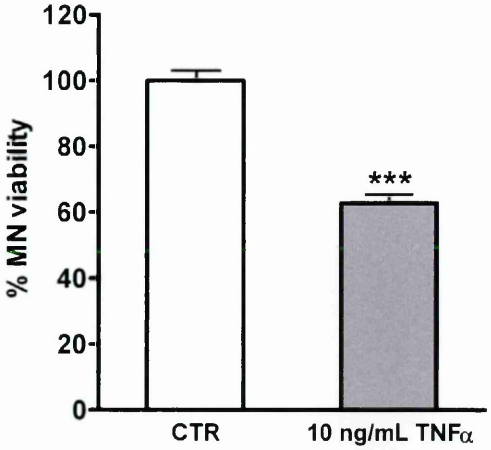


Figure 21. TNF-α conditioned glia reduces motor neuron survival in cocultures.

Purified motor neurons were seeded over a control confluent glial layer (CTR) or over a glial layer pre-treated with 10 ng/mL TNF-a for 72 h before the establishment of cocultures. 1 week old cocultures were then stained by SMI32 and positive motor neurons were counted. Bars represent the mean percentage ± SD of SMI 32-positive cells in each treatment condition.

*** $p < 0.001$ vs CTR. ANOVA and Tukey test.

7.1.2 TNF receptor expression in motor neuron cultures

Since TNF-α exerted neurotoxicity only when mature glial cells were in the cultures, we verified the expression of TNF receptors in cocultures (4 weeks old glia) or in mixed anterior horn cultures (1 week old glia). In experiments with cocultures, motor neurons were plated over conditioned (pre-treated with 10 ng/mL TNF-α for 72 hours; Fig 22 E, F, G, H) or control (not treated; Fig 22 A, B, C, D) mature glial layers. Then, they were double-stained by SMI32 (red, Fig 22 A, E, C, G) and TNFR-1 (Fig 22 B, F) or TNFR - 2 (Fig 22 D, H) antibodies. Both TNFR-1 and -2 showed a low immunostaining in motor neurons, but were highly localized in SMI32-negative cells (Fig 22 B, D), suggesting that glial cells express the two receptors. Conditioned glial cells did not affect this trend of expression and both TNFR-1 (Fig 22 F) and -2 (Fig 22 H) antibodies gave a similar pattern of immunofluorescence, with SMI32-negative cells being intensively stained.

In another set of experiments, mixed anterior horn cultures were double-stained by GFAP (revealing the astrocytes) and by the TNFR-1 antibody. Despite the presence of a high number of GFAP-positive glial cells (Fig 22 I, in red), the staining of TNFR-1 could not be revealed (Fig 22 J), suggesting that in this culture condition (1 week of *in vitro* aging) glial cells did not yet express TNFR-1.

Conclusions

We reported here evidence that the *in vitro* ageing of the glial population is the determinant for eliciting TNF-α-mediated neurotoxicity and protection against the AMPAR-mediated motor neuron death (when comparing the results obtained in mixed anterior horn cultures to those in cocultures). We revealed high immunoreactivity of TNF receptors in 4 week old glial cells (used in cocultures), while we could not obtain evidence for the presence of the receptors on glial cells grown in vitro for 1 week

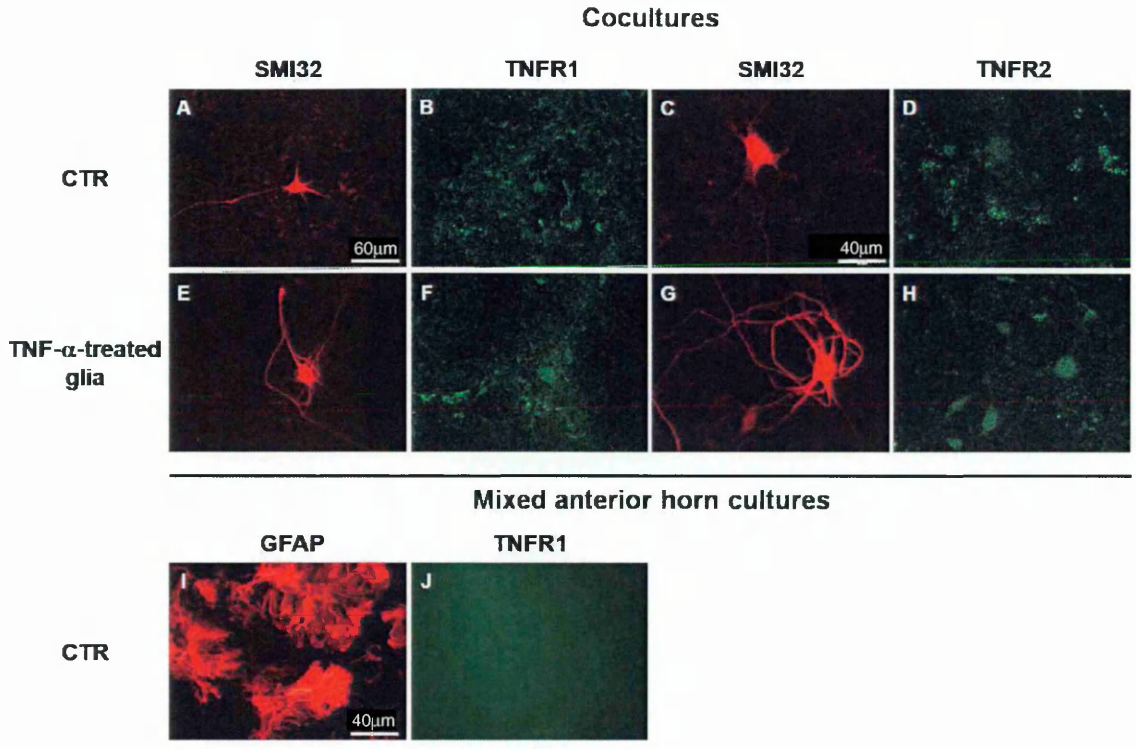


Figure 22. TNFRs are expressed in cocultures, but not in mixed anterior horn cultures.

Cocultures were established over control (not treated) or 10 ng/mL TNF-α-treated glial layer. After 1 week from the establishment of cocultures, they were double-stained by SMI32 (red, A, C, E, G) and TNFR1 (green, B, F) or TNFR2 (green D, H). Both the receptors are expressed in cocultures by motor neurons and by SMI32-negative cells, independently from the presence of control or conditioned glia.

In picture I and J, 1 week old mixed anterior horn cultures were double-stained by GFAP (red, I) and TNFR1 (green, J). TNFR1 is not expressed in this culture condition.

(mixed anterior horn cultures). Consistently, when tested on motor neuron survival, even at high concentrations (100 ng/mL) TNF-α was ineffective in mixed anterior horn cultures. On the other hand, when a mature glial layer was present (cocultures) TNF-α treatment induced motor neuron death both if present in the incubation medium and after withdrawal from previous 72 hour pre-treatment. Experiments with conditioned glia (pre-treated with the cytokine) demonstrated that the activation of the glial cells by 10 ng/ml TNF-α induced a neurotoxic phenotype that affected motor neurons subsequently added to the glial cultures even if they were not directly exposed to the cytokine.

We also documented a significant interaction between the effect mediated by TNF-α and the AMPAR-dependent toxicity in cocultures, while no interaction was present in mixed anterior horn cultures. We showed, in fact, that 10 ng/mL TNF-α exerted neuroprotective effect by significantly reducing the motor neuron death induced by different AMPA concentrations. Also the TNF-α neurotoxicity was significantly reduced by co-treatment with the AMPAR agonist.

Taken together these data are in accordance with the results described in the literature demonstrating the central role of glia in mediating TNF-α-dependent neurotoxicity and protection against excitotoxic insults, and clearly indicate that this effect is dependent on the presence of mature glial cells expressing the TNF receptors. Activated glial cells may in turn induce a toxic effect on neurons probably by the release of toxic mediators, or, alternatively, trigger neuroprotective mechanisms such as the TNF-mediated stabilization of neuronal $[Ca^{2+}]i$ (Cheng *et al.* 1994), suggesting the importance of the strict cross-talk between neurons and glia.

7.2 Interleukin-8

Background

The presence of chemokine receptors has been described in the CNS (for review, see (Cartier *et al.* 2005). In particular, the chemokine receptor CXCR2, which mediates the recruitment and activation of polymorphonuclear (PMN) and other leucocytes by IL-8 at the site of tissue damage during inflammatory reactions, is the most strongly expressed chemokine receptor on neurons and is upregulated in Alzheimer disease (Horuk *et al.* 1997, Xia & Hyman 2002). CXCR2 is also expressed on oligodendrocytes and its ligands are present in multiple sclerosis lesions, meaning that there are CXCR2-mediated effects also in a disease that is not typically associated with PMN infiltration and suggesting that chemokines may do more than just mediate recruitment of inflammatory cells (Omari *et al.* 2006, Omari *et al.* 2005) . The few data published on the trophic or toxic effects of CXCR1/2 and their ligands in neuronal cultures are controversial. IL-8 enhanced the survival of hippocampal cultures in vitro, possibly by an indirect effect mediated by increased astroglial and microglial proliferation (Araujo & Cotman 1993). IL-8 also showed neuroprotective activity against NMDA and β-amyloid-induced toxicity in mixed cortical cultures (Bruno *et al.* 2000). A protective effect of IL-8 against apoptosis induced by low potassium-containing medium was reported in cerebellar granule cells (Limatola *et al.* 2002, Limatola *et al.* 2000). However, other reports have shown that IL-8 has detrimental effects such as induction of Tau phosphorylation (Xia & Hyman 2002) and of pro-apoptotic proteins in primary neurons (Thirumangalakudi *et al.* 2007).

To study the role of chemokines acting via CXCR2 in motor neuron diseases, we investigated the effect of the CXCR2 ligand MIP-2 (CXCL2; homologue of IL-8 in rodents) on primary motor neuron cultures, both as mixed anterior horn cultures and as

purified motor neurons from rat embryos. To specifically address the role of CXCR2, we also used motor neurons from wild-type and CXCR2-deficient mice.

7.2.1 IL8 receptor CXCR1/2 expression on motor neurons

We verified the presence of the two IL-8 receptor homologues in rodents (i.e. CXCR1 and CXCR2) on motor neurons in mixed anterior horn cultures. Figure 23 shows the double-staining of mixed anterior horn cultures labelled with SMI32, selectively identifying motor neurons, and with the anti-CXCR1 or anti-CXCR2 antibody. Both antibodies labelled the entire cell population, i.e. neurons and glial cells. However, while the staining for CXCR2 (Fig 23 F) was completely prevented by incubation with an excess of the specific blocking peptide (Fig 23 H), we could not demonstrate the specificity of CXCR1 staining (Fig 23 B) with its blocking peptide (Fig 23 D). The anti-CXCR2 antibody intensely stained glial cells and seemed to label the entire motor neuronal cell (Fig. 23 E, F), both in the soma and in the arborizations.

7.2.2 Neurotoxic effect mediated by CXCR2 activation

We investigated the effect of recombinant rat MIP-2 in mixed anterior horn cultures. Figure 24 shows that MIP-2 induced dose-dependent neurotoxicity triggering about 50% motor neuron death at 12.5 nM. The boiled protein had no effect even at the highest concentration (12.5 nM), indicating that the neurotoxic effect was not due to a contaminant (Fig 24). MIP-2, at 12.5 nM, was toxic also to purified motor neuron cultures (41 $\pm$ 6.5 % death, $p<0.001$; fig 25) indicating that the toxicity was due to a direct effect on motor neuron. Experiments performed with the human chemokine (IL-8) gave similar results, not significantly different from those obtained with MIP-2 (not shown). To see whether the in vitro neurotoxicity of MIP-2 was specifically mediated by CXCR2, we studied the effect of chemokine on mixed anterior horn cultures

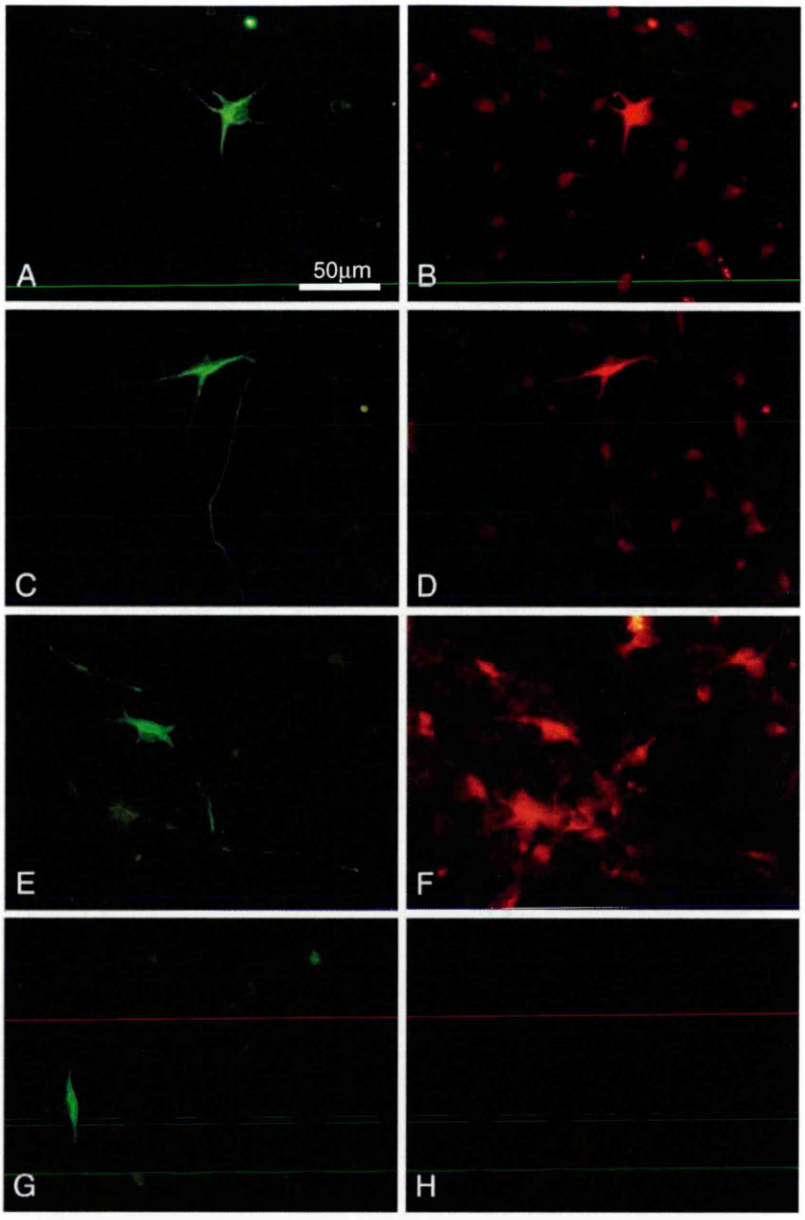


Figure 23. CXCR1 and CXCR2 distribution in motor neurons.

Mixed anterior horn cultures were double-stained with SMI32 (green, A , C, E , G) and the specific antibodies for CXCR1 or CXCR2 (red, B , D , F , H). CXCR1 and CXCR2 were revealed by primary antibody sc-23811 (B , D) or sc-683 (F , H) respectively, as described in Methods.

D, H: CXCR1 and CXCR2 staining after incubation with an excess of specific blocking peptides.

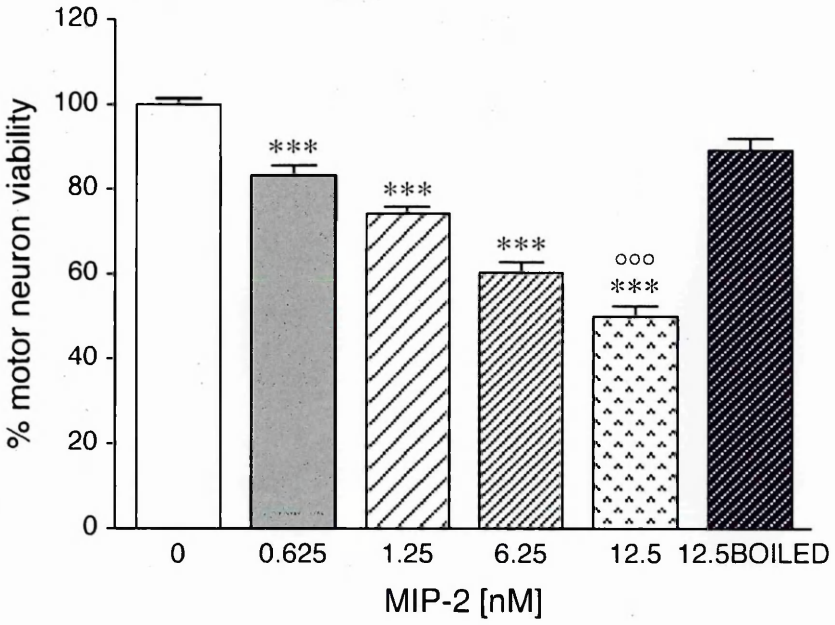


Figure 24. Dose-response effect of MIP-2 on motor neuron viability in mixed anterior horn cultures.

Viability of SMI32-positive motor neurons in mixed anterior horn cultures after 48 hour treatment with different MIP-2 concentrations. Data are expressed as the percentage of SMI32-positive cells normalized over controls, and are means $\pm$ SD of 8 replicates from 4 independent experiments.

*** $p < 0.001$ vs control; ††† $p < 0.001$ vs boiled solution, ANOVA and Tukey test.

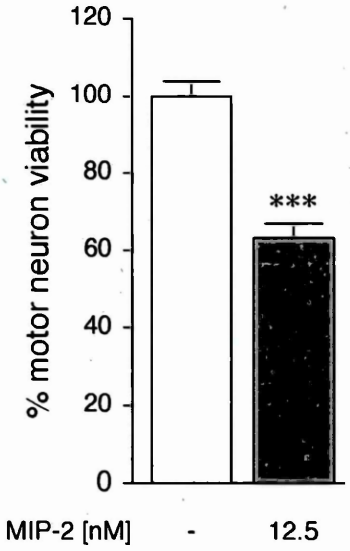


Figure 25. Toxic effect of MIP-2 on motor neuron viability in purified motor neuron cultures.

Viability of SMI32-positive motor neurons in purified motor neuron cultures after 48 hour treatment with 12.5 nM MIP-2. Bars represent the percentage of SMI32-positive cells normalized over controls, and are means ± SD of 8 replicates from 4 independent experiments.

*** $p < 0.001$ vs control (no MIP-2), ANOVA and Tukey test.

prepared from individual CXCR2 –/– or control (both CXCR2 –/+ and CXCR2 +/+) mouse embryos. As expected, 12.5 nM MIP-2 induced motor neuron death in cultures from wild-type mice. Primary cultures from the spinal cords of CXCR2-deficient mouse embryos developed like those from wild-type mice and motor neurons did not differ in terms of morphology or SMI32 staining. However, 12.5 nM MIP-2, incubated for 48 h, was not toxic for motor neurons from CXCR2 knock-out embryos, but induced 30-40% death in those from control or heterozygous embryos (Table II).

TABLE II. Effect of MIP-2 on mouse mixed anterior horn cultures (% of motor neuron viability)

	MIP-2	MIP-2 + reparixin
Wild type mice (+/+)	68 ± 22 (5) *	86 ± 14 (3) §
(+/-) mice	58 ± 11 (10) **	95 ± 19 (5) §
CXCR2 KO mice (-/-)	100 ± 16 (3)	--

Mixed anterior horn cultures from single mouse embryos were incubated for 48 h with 12.5 nM MIP-2, in the presence or absence of 10 μM reparixin.
Data represent means ± SD of individual culture preparations, the number of replicates is given in parenthesis.
* $p< 0.05$, ** $p<0.01$: different from -/- mice (ANOVA and Tukey test)
§ $p<0.0021$, different from MIP-2 alone (ANOVA paired test)

Conclusions

These results are the first demonstration of a toxic effect of a CXCR2 ligand on motor neurons. We showed MIP-2 dose-dependent neurotoxicity both in mixed anterior horn cultures and in purified motor neuron cultures, indicating a direct toxic effect of CXCR2 activation on the motor neuron, without the involvement of glial cells. We confirmed the presence of CXCR2 receptor on motor neurons, but could not document the specific immunostaining for CXCR1, in agreement with earlier reports showing CXCR2, not CXCR1, being expressed at high levels in some CNS regions, including motor neurons in the anterior horn of the human spinal cord (Horuk *et al.* 1997). The lack of effect of MIP-2 in mixed anterior horn cultures from CXCR2-deficient mice further indicated that the toxicity was specifically mediated by this receptor.
This finding supports the hypothesis that high IL-8 levels during inflammatory reactions could exert also a direct neurotoxicity mediated by CXCR2 on motor neurons.

Chapter 8 - Protein aggregation and excitotoxicity: the dual role of α-synuclein

α-Synuclein is a small (14 kDa) protein mainly expressed in the brain and predominantly concentrated in presynaptic nerve terminals. At physiological concentrations α-synuclein is an unfolded protein with no ordered secondary structure but it is well known that it can undergo polymerisation into fibrils associated with the formation of toxic aggregates (Moran *et al.* 2001). Overexpression of α-synuclein resulted in cytotoxicity while at nanomolar concentrations it protected neurons against serum deprivation, oxidative stress and excitotoxicity. Stable α-synuclein transfected neuronal cell lines have been shown to be protected from toxic insults such as H_2O_2 and the mitochondrial toxin MPP+ (Hashimoto *et al.* 2002, Jensen *et al.* 2003). NGF-differentiated PC12 cells have been shown to be protected from H_2O_2, 6-OH DA and serum deprivation if previously incubated with the fusion protein TAT-α-synuclein (Albani *et al.* 2004). Finally α-synuclein protected rat primary cortical and hippocampal neuronal cells from death induced by serum deprivation and SHSY-5Y, GT1-1 and NGF-differentiated PC12 cells againstH_2O_2, glutamate and serum deprivation treatments (Seo *et al.* 2002).

α-synuclein aggregates are associated with several neurodegenerative disease including Parkinson's disease, Alzheimer's disease, Lewy body dementia, multiple system atrophy and recently also to ALS (Bennett 2005). Aggregates of α-synuclein have been observed in neuronal spheroids, astrocytes, Schwann cells and in corticospinal axon tract fibers and glia in brain and spinal cord of ALS patients (Doherty *et al.* 2004, Mezey *et al.* 1998). In addition an increased expression of α-synuclein has been detected in the anterior horn of the spinal cord of SODG93A transgenic mice, an animal model of ALS (Chung *et al.* 2003).

On the basis of such evidence, suggesting the involvement of α-synuclein in the pathogenesis of ALS, we investigated the effect of the protein in motor neuron degeneration by using purified cultured motor neurons.

8.1 α-Synuclein insertion and effect on motor neurons

On the seventh day in vitro (DIV 7) cultures were incubated for 18h with the fusion protein TAT-α-synuclein, generated from the insertion of the sequence containing the minimal translocation domain of the HIV1 protein TAT in frame before the N-terminal of the corresponding α-synuclein cDNA (Albani *et al.* 2004). Motor neurons treated with 100 nM or higher TAT-α-synuclein concentration showed a dose-dependent decrease in viability ($p < 0.001$ at all α-synuclein > 100 nM concentrations tested, Fig 26 A). Lower concentrations (tested up to 10 nM) did not affect motor neuron viability. From the dose-response curve (Fig 26 B) the calculated EC50 was 107 $\pm$ 22 nM. α-Synuclein internalization was then investigated by immunofluorescent assay using the primary antibody anti-α-synuclein. Immunofluorescence confirmed the typical fine punctate α-synuclein staining (Fig 27 A) previously observed in neuronal cells by Quilty and colleagues (Quilty *et al.* 2003). Even in control conditions, motor neurons were intensively labelled for α-synuclein throughout the cell body and processes with a patch-like distribution typical of the cytoskeleton constitutive proteins. For this reason it was not possible to quantify the further contribution of the exogenous α-synuclein, even when a lower concentration of primary antibody or the DAB staining was used (not shown). We obtained very similar results by using the anti-oligomer antibody which showed the same punctate staining observed with the antibody for the monomeric form of the protein, with no differences between control and α-synuclein-treated motor neurons (Fig 27 B). Although it was not a specific staining of α-synuclein, we clearly

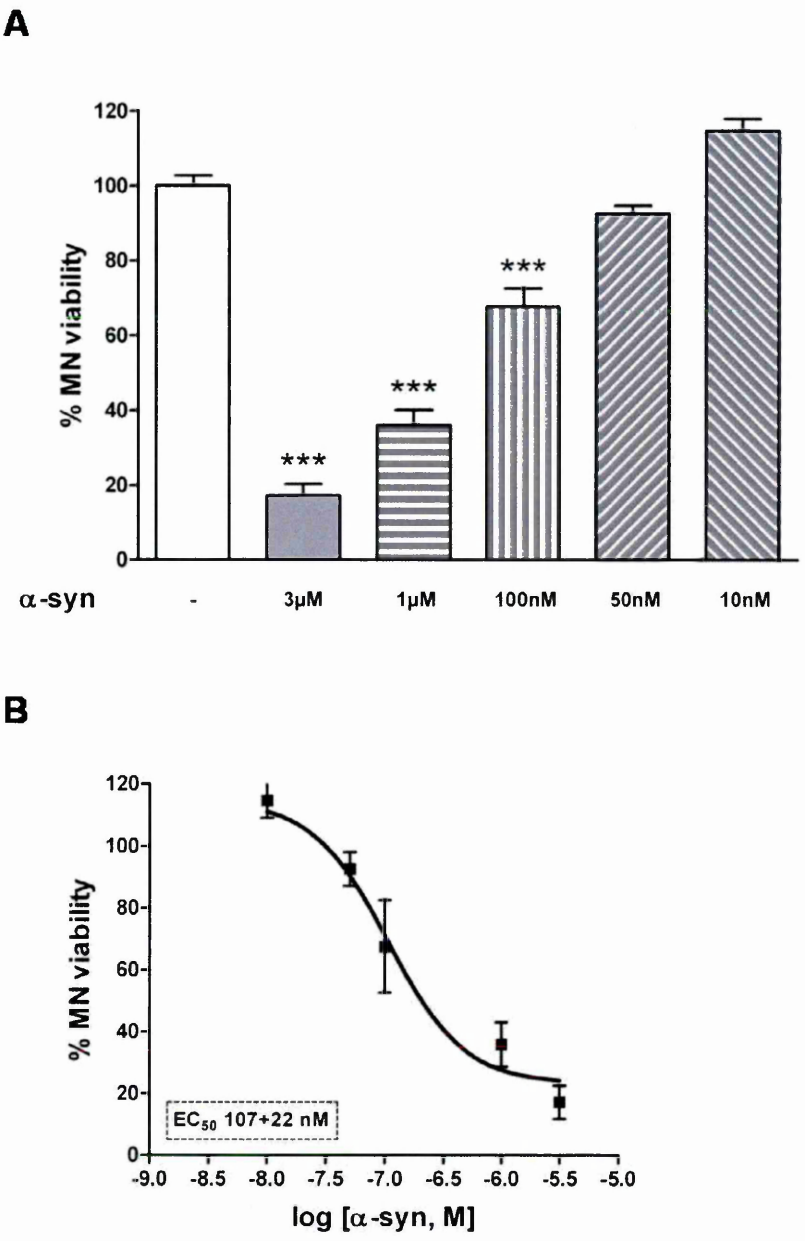


Figure 26. Dose-dependent neurotoxicity of α-synuclein.

After 1 week in culture, purified motor neurons were exposed to different concentrations of the fusion protein TAT-α-synuclein for 18 h and then stained by SMI32. Panel A: bars represent the mean percentage ± SD of SMI 32-positive cells obtained from 6 replicates for each condition. * * * $p < 0.001$ vs control (no α-syn), ANOVA and Tukey test. Panel B shows the dose-response curve of α-syn. Data were fitted with the one site competition equation built in GraphPad Prism 5.01; each point represents mean ± standard deviation of 6 replicates from 3 independent experiments. The calculated EC50 is reported.

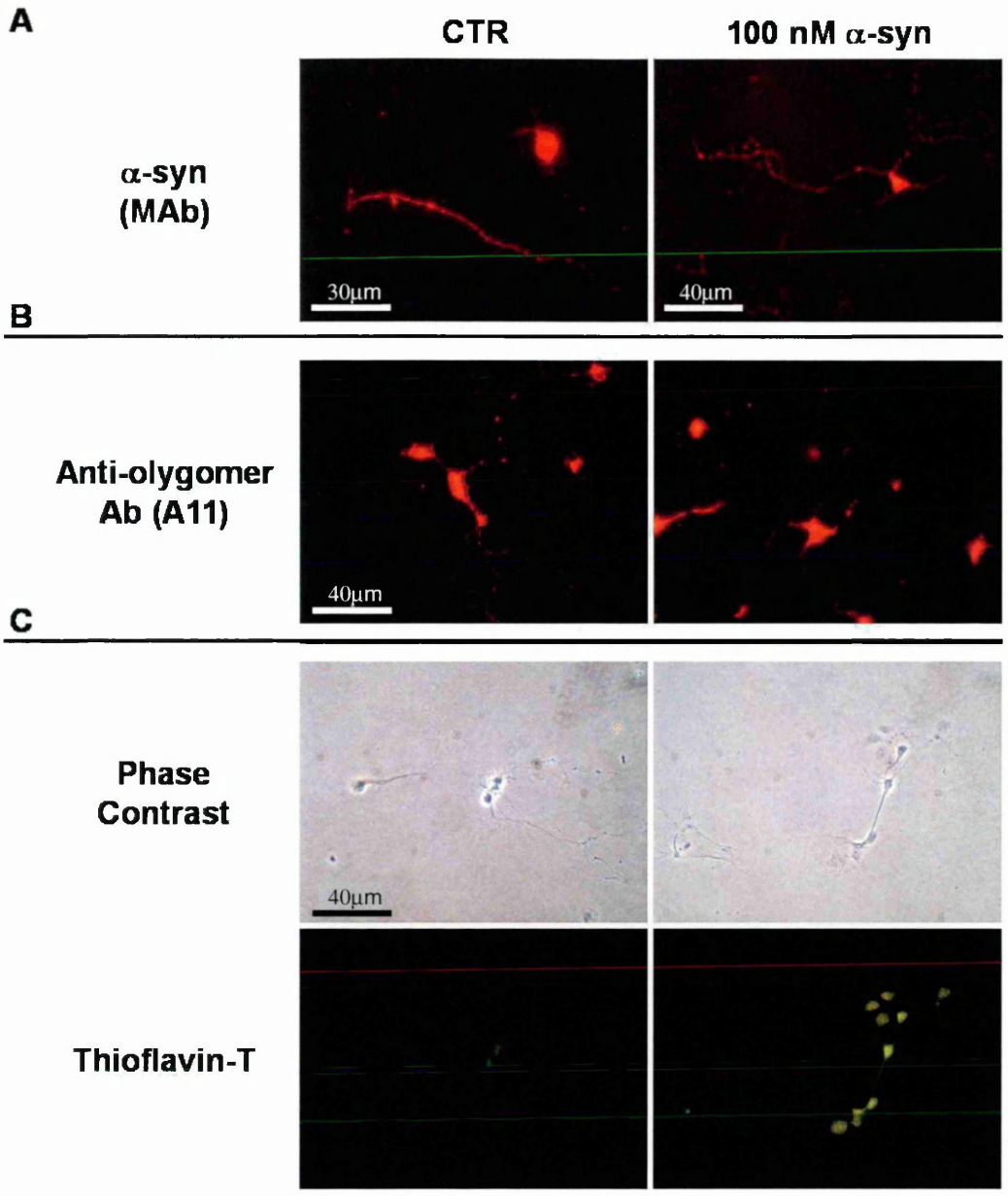


Figure 27. Intracellular α-synuclein visualization.

After 1 week in culture, purified motor neurons were exposed to 100 nM α-syn for 18h and then stained by different immunocytochemical assay. Panel A: motor neurons stained by the α-syn monoclonal antibody for the detection of monomeric α-syn form. Panel B: motor neurons stained by the A11 antibody for the olygomeric α-syn form. Panel C: motor neurons (in phase contrast, upper row) labeled by the Thioflavin-T assay (lower row), revealing the amyloid aggregates. Only the Thioflavin-T assay revealed increased aggregates after 100 nM α-syn treatment.

identified increased fluorescent signal in α-synuclein-treated motor neurons compared to control by the thioflavin-T binding assay (Fig 27 C), which detects amyloid aggregates, demonstrating intracellular protein accumulation.

8.2 Protective effect of low α-synuclein concentration

Since previous evidence demonstrated that nanomolar concentrations of α-synuclein resulted in neuroprotection against different cytotoxic stimuli, we also examined in our model whether low α-synuclein concentrations could preserve motor neurons from death. Thus we investigated the effect of the protein against serum deprivation, H_2O_2 or kainate induced toxicity. Purified motor neuron cultures were pre-incubated with 50 nM TAT-α-synuclein for 3h on DIV 7 and then they were co-treated with TAT-α-synuclein and 100 μM H_2O_2 or 50 μM kainate for 18 or 48h respectively (treatments known to induce about 50% of motor neuron death in our experimental condition). 50 nM TAT-α-synuclein protected motor neurons from H_2O_2 - mediated toxicity (approximately 20% viability increase, Fig 28). Quite surprisingly, α-synuclein, at the same concentration shown to bc effective on oxidative stress-dependent toxicity, did not protect motor neurons from excitotoxicity, since the percentage of motor neuron death induced by 50 μM kainate was not significantly altered by co-treatment with the fusion protein (Fig 28). For serum deprivation, after the pre-incubation with TAT-α-synuclein cultures were treated with the protein dissolved in serum free-medium for 18 hours. Here different nanomolar concentrations of the protein were tested, but none exerted any neuroprotective effect (Fig 29).

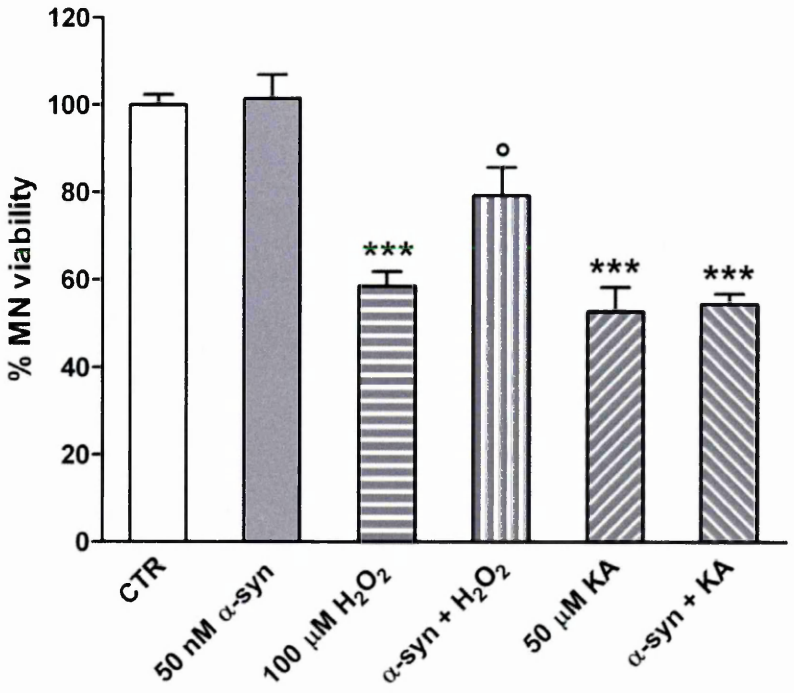


Figure 28. α-Synuclein is protective against H_2O_2- but not kainate-induced motor neuron death.

After 1 week in culture, purified motor neurons were pre-treated by 50 nM α-syn for 3 h and then co-incubated with a solution of the protein added of 100 μM H_2O_2 or 50 μM KA for further 18 h or 48 h respectively. Then, motor neurons were stained by SMI32. Bars represent the mean percentage ± SD of 6 replicates from 3 independent experiments.

* * * $p < 0.001$ vs control; ° $p < 0.05$ vs 100 μM H_2O_2 alone, ANOVA and Tukey test.

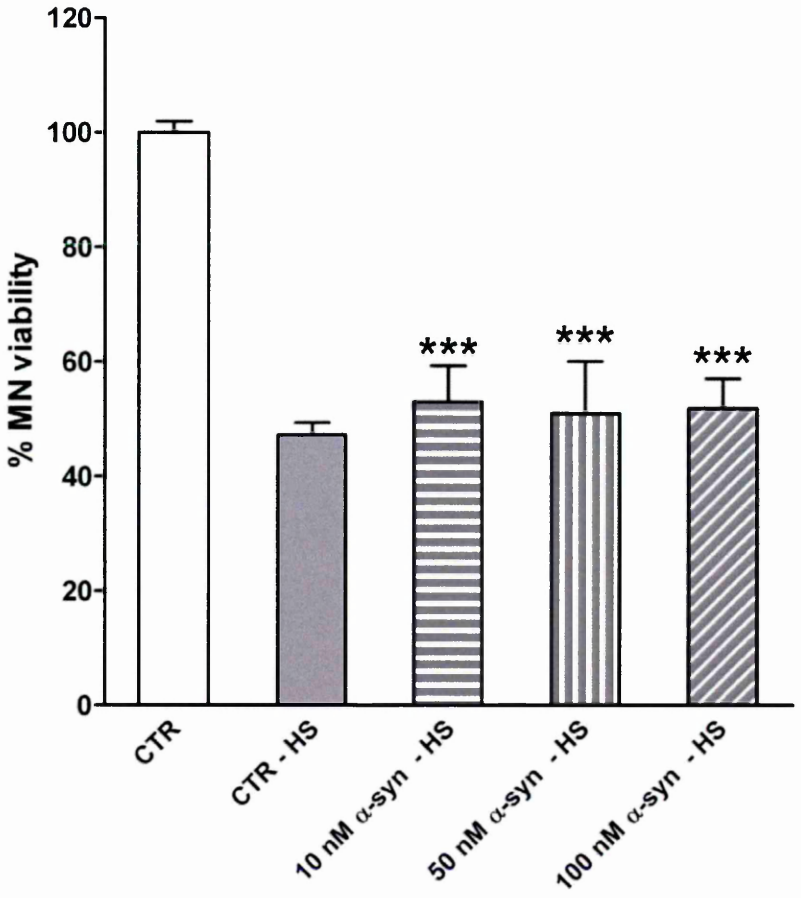


Figure 29. α-Synuclein does not affect the motor neuron death occurring after serum deprivation.

After 1 week in culture, purified motor neurons were pre-treated by 50 nM α-syn for 3 h and then incubated with a solution of the protein diluted in medium free of serum and growth factors for further 18 h. Then, motor neurons were stained by SMI32. Bars represent the mean percentage ± SD of 6 replicates from 3 independent experiments.

* * * $p < 0.001$ vs control, ANOVA and Tukey test.

Conclusions

Our experiments provide evidence to support a dual concentration-dependent effect of α-synuclein in cultured motor neurons, in accordance with what has been reported in other cell cultures (Albani *et al.* 2004, Seo *et al.* 2002). Our data indicated that TAT-α-synuclein displayed neuroprotective effect against oxidative stress at 10 nM. At the same concentrations effective against H_2O_2, TAT-α-synuclein was not able to counteract cell death induced by 50 μM kainate or serum deprivation. This suggests that the α-synuclein-dependent protective effect in motor neurons could be mediated by specific events which are mainly involved in oxidative stress conditions but do not have a fundamental role in excitotoxicity or serum deprivation. Conversely, as the concentration of the protein increased, a toxic effect was shown and motor neuron viability significantly decreased with an EC50 about 100 nM. We failed to specifically verify whether this toxicity was dependent on the accumulation of the protein in motor neurons, although amyloid aggregates were likely to be present in the toxic conditions (100 nM α-synuclein for 18 hours), as revealed by the thioflavin-T assay. Whether the toxic effect revealed in primary motor neuron cultures is dependent on protein accumulation inside these specific cells remains to be elucidated.

Chapter 9 - Pharmacological approaches

An important aim of this research project was to test pharmacological compounds that might exert neuroprotection by interfering with the mechanisms of death that I analysed *in vitro*. Thus, the results obtained in primary motor neuron cultures brought us to test agents acting at different steps of the death pathways or on specific receptors with neurotoxic features. In particular we obtained interesting evidence for a protective effect by two classes of compounds: 1) erythropoietin (EPO), a hematopoietic growth factor which prevented the apoptotic cell death induced by AMPAR agonists, and its non-erythropoietic derivatives (CEPO, ASIALO-EPO and HBP) 2) reparixin, an orally active CXCR1/2 inhibitor counteracting the toxicity induced by MIP-2.

9.1 Erythropoietin and its derivatives

Background

EPO is a glycoprotein originally identified as the regulator of erythroid progenitor cells. EPO is induced in hypoxic conditions through the hypoxia-inducible factor 1 (HIF-1) transcription factor (Semenza & Wang 1992). Systemically administered EPO crosses the blood brain barrier (Brines *et al.* 2000) and showed neuroprotection in different models of neurodegenerative disease, including experimental autoimmune encephalomyelitis (EAE; Agnello *et al.* 2002, Savino *et al.* 2006), cerebral ischemia (Siren *et al.* 2001), and diabetic neuropathy (Bianchi *et al.* 2004). Its mechanism of action is not completely understood: in addition to its anti-apoptotic effect (Siren *et al.* 2001), EPO inhibits CNS inflammation (Agnello *et al.* 2002, Villa *et al.* 2003), enhances neurogenesis in animal models of stroke and EAE (Wang *et al.* 2004, Zhang *et al.* 2005), and augments BDNF expression in vivo and in vitro (Viviani *et al.* 2005, Wang *et al.* 2004). Thus, in addition to promoting a hematopoietic effect, EPO showed protective effects in different in vitro and in vivo models of neurodegeneration. Since

chronic administration of EPO results in an increase in the hematocrit, which could increase the risk of thrombosis, different non-erythropoietic molecules retaining the neuroprotective activities of EPO have been derived from EPO. One of these molecules, carbamylated EPO (CEPO), has proven effective in animal models of stroke, EAE, spinal cord injury, and diabetic neuropathy (Leist *et al.* 2004). Unlike EPO, CEPO does not bind the erythropoietic receptor EPOR (Leist *et al.* 2004), and its neuroprotective action appears to require the common β chain of IL-3/IL-5/GM-CSF receptor (also known as CD131) (Brines *et al.* 2004), which can functionally associate with EPOR (Jubinsky *et al.* 1997). Another non-erythropoietic EPO derivative is asialo erythropoietin (ASIALO-EPO), which, although binding to EPOR, has a short half-life and does not increase the hematocrit (an activity that requires persistent circulating levels of EPO) but also retains neuroprotective activities in vivo (Erbayraktar *et al.* 2003).

HBP is a synthetic peptide, fragment of EPO, lacking the regions of EPO that interact with EPOR, but mimicking the helical structure of the full-length protein that interacts with CD131 (Brines *et al.* 2008). In fact the peptide contains the amino acid sequence corresponding to helix B (residues 58–82) which is needed for the binding to CD131.

In this study we investigated the effect of EPO on motor neuron death elicited by two different stimuli that seem to play a relevant role in inducing or enhancing the progressive loss of motor neurons in ALS: neurotrophic factors withdrawal and AMPAR-mediated excitotoxicity. Moreover we tested if EPO analogues with no effect on hematopoiesis (ASIALO-EPO, CEPO and HBP) retain the neuroprotective effect of the parent compound.

9.1.1 EPO is neuroprotective against apoptotic death induced by low AMPAR agonist concentrations or by serum/BDNF deprivation

We first verified the presence of EPOR in mixed anterior horn cultures. The double staining of SMI32 and the specific antibody for EPOR revealed that EPOR was present both on motor neurons (Fig 30 C, merge staining) and on SMI32-negative cells (Fig 30 C, red label). In particular, it stained motor neurons both on cell bodies and arborizations, and seemed to be located both in the membrane and in the cytosol. To assess if repeated EPO treatments modify the expression or the distribution of EPORs on motor neurons, purified cultures were treated with EPO (2.5 pmol/mL) for 5 days. The staining revealed no difference in the intensity and distribution of EPORs in EPO-treated cells compared with vehicle-treated cells (not shown), indicating that EPO, in the experimental conditions tested, neither downregulated nor upregulated EPOR.

For experiments on the effect induced by EPO treatment, cell death was induced on the sixth day in culture by incubating mixed anterior horn cultures for 48 hours with 5μM kainate (low AMPAR agonist concentration) or 1μM AMPA (high AMPAR agonist concentration). EPO (2.5 pmol/mL) or vehicle was added to the cultures 72 hours before induction of cell death, and treatment continued for the 48 hours of exposure to the AMPAR agonists. The viability of motor neurons in mixed anterior horn cultures was significantly reduced after treatment with 5 μM kainate and returned to control values in cells treated with 2.5 pmol/mL EPO (Fig 31 A). The survival of untreated cultures exposed to EPO was above control values (128 $\pm$ 38 n.s.; Fig 31 A), demonstrating the neurotrophic effect of EPO, possibly related to reduced spontaneous apoptosis.

1 μM AMPA caused 50% of cell death and EPO was not effective at protecting motor neurons from this excitotoxicity stimulus (Fig 31 A). These results indicate a selective effect of EPO against the AMPAR-dependent apoptotic cell death. The effect of EPO was concentration-dependent between 0.25 and 2.5 pmol/mL (concentration that

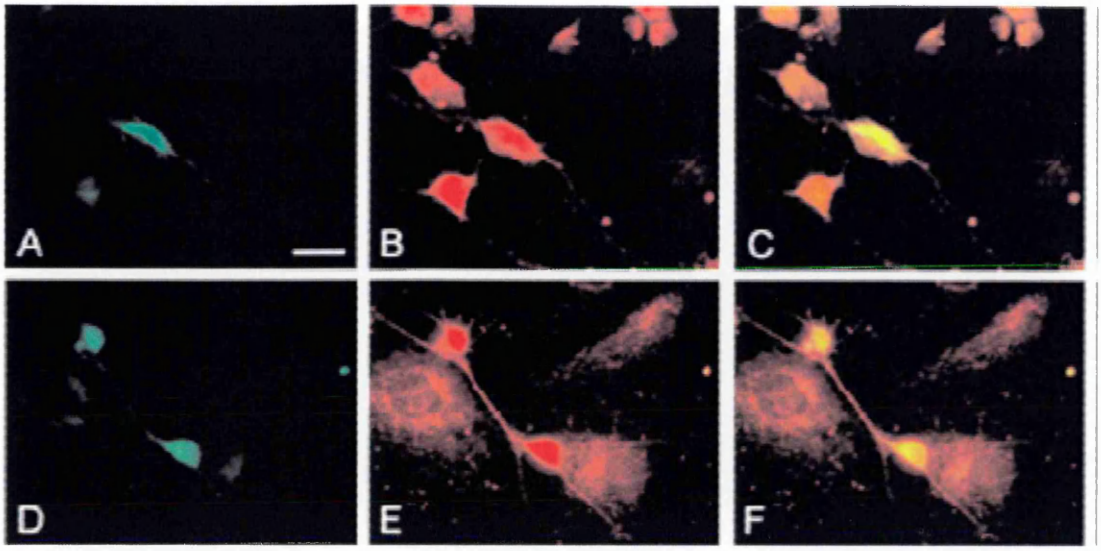


Figure 30. Motor neurons express EPO and CD131 receptors.

Mixed anterior horn cultures were double-stained by SMI32 (green, A, D) and the specific antibody against EPOR (B) or the β chain common to IL-3, IL-5, and GM-CSF receptors (E). C and F are the merged pictures. Co-incubation with an excess of the respective blocking peptides completely abolishes the specific staining of anti-EPOR and anti–IL-3R β antibodies (not shown). Scale bar: 25 μm.

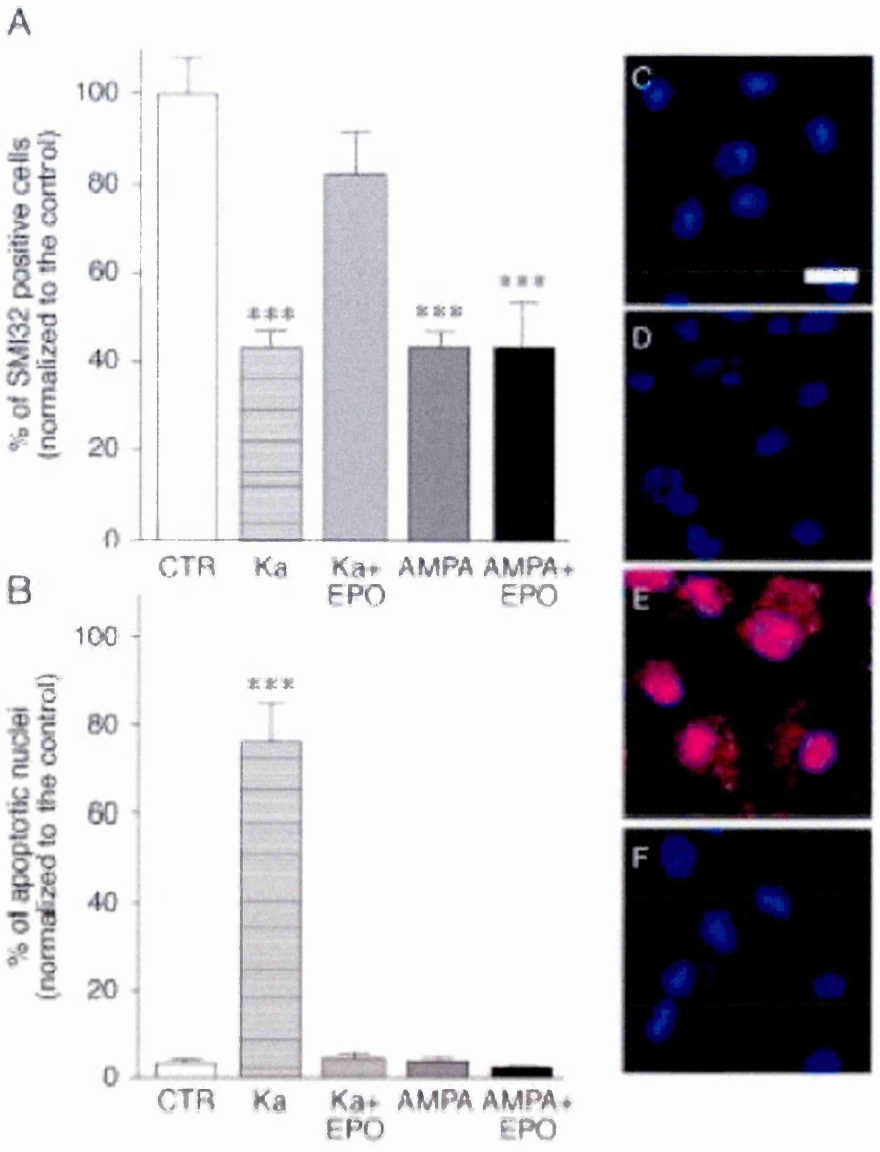


Figure 31. EPO counteracts the motor neuron death induced by low, but not the higher, AMPAR agonist concentrations

Panel A : mixed anterior horn cultures pre-treated with 2.5 pmol/mL EPO for 72 h and then co-treated with the cytokine and 5 μM KA or 1 μM AMPA for further 48 h. *** $p<0.001$ vs control.

Panel B: mixed anterior horn cultures pre-treated with 2.5 pmol/mL EPO for 72 h and then co-treated with the cytokine and 5 μM KA or 1 μM AMPA for further 18 h. *** $p < 0.001$ vs other treatment condition.

C-F: Syto59 (blue) and PI (red) double-staining in purified motor neuron cultures. Control cells are impermeable to PI and do not show DNA fragmentation (C). Kainate treated cells undergo to a massive DNA fragmentation and nuclear shrinkage thus remaining impermeable to PI (D). AMPA treated motor neurons showed a massive PI penetration accompanied by nuclear enlargement (E). EPO completely counteracts the effect produced by kainate treatment (F). Scale bar C-F, 20 μm.

produced full protection against kainate toxicity), with an ED50 of about 1.25 pmol/mL. Figure 32 shows SMI 32–positive motor neurons in mixed anterior horn cultures exposed to different treatments. After 72+48 hour treatment, 2.5 pmol/mL EPO produced a clear neurotrophic effect, increasing the neurite outgrowth and the number and differentiation of motor neurons (Figure 32 B) compared to control conditions (Fig 32 A). EPO was also neuroprotective when associated with 5 μM kainate (Figure 32 D) restoring motor neurons in number and morphology, which were highly compromised by the treatment with the agonist alone (Fig 32 C).

Since in this experimental condition only low AMPAR concentrations, but not the higher, induced caspase activation and nuclear fragmentation (see Chapter 6, sections 6.2/6.5), further experiments were performed to investigate the effect of EPO on the specific apoptotic intracellular events.

After 18 hours, the high percentage of SMI32-positive motor neurons showing nuclear fragmentation induced by 5 μM kainate treatment was significantly reduced by pre-treatment with 2.5 pmol/mL EPO (Fig 31 B). 1μM AMPA treatment confirmed that no significant nuclear fragmentation was triggered compared to control condition, and EPO treatment did not affect this result (Fig 31 B).

In order to define whether DNA fragmentation was actually related to an exclusively apoptotic mechanism, double staining experiments using the fluorescent nucleic acid dyes SYTO 59 and propidium iodide (PI) were done. Fig 31 C shows the pattern of staining for PI (red) and SYTO 59 (blue) in purified motor neurons in basal condition. As for Hoechst 33258 staining the nuclei in control condition displayed a physiological morphology with homogeneous distribution of the nuclear dye, no PI staining was observed in these cells (Fig 31 C). Although low concentration kainate treatment produced a marked nuclear fragmentation and shrinkage (Fig. 31 D), motor neurons did not lose membrane integrity, as indicated by the lack of PI staining. Following the

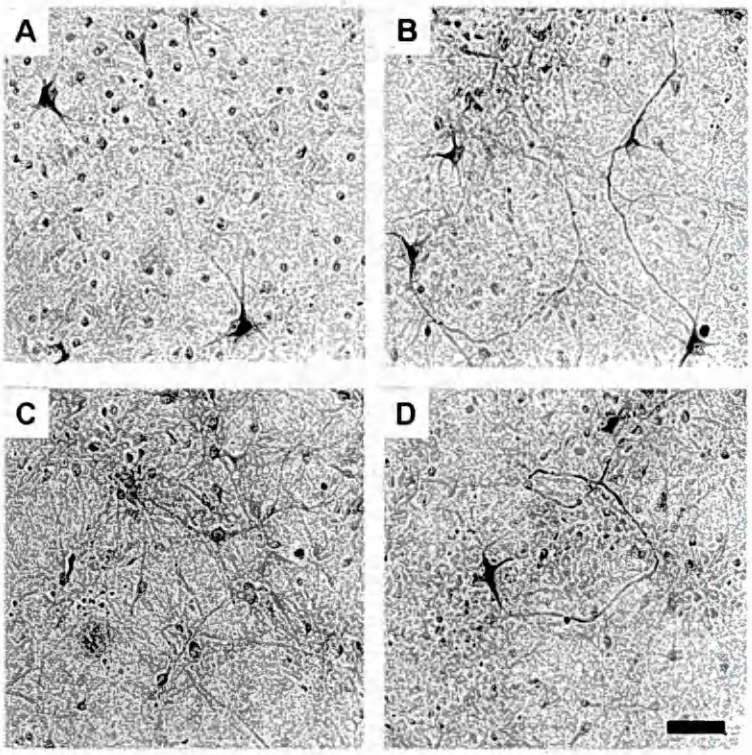


Figure 32. Neurotrophic and neuroprotective effect of EPO on SMI 32-positive motor neurons in mixed anterior horn cultures.

Mixed anterior horn cultures stained by SMI32 after different treatments. A: motor neurons in control cultures, well defined morphologically. B: mixed anterior horn cultures after treatment with EPO (2.5 pmol/mL) alone for 5 days. Large cell bodies with long axons and increased number of SMI32-positive motor neurons were shown. C: cultures treated for 48 h with 5 μM kainate. D: effect of EPO (added 72 h before and during the 48 h of exposure to kainate) on cultures treated with kainate.

Scale bar = 40 μm.

treatment with 1 μM AMPA, purified motor neuron exhibited marked cytoplasmic PI positivity and an increased nuclear size without fragmentation (Fig 31 E). Both are common features of necrotic death. EPO counteracted kainate-induced nuclear fragmentation of motor neurons (Fig 31 F) but was ineffective when a non-apoptotic cell death was triggered with high AMPAR agonist concentrations (not shown).

For the detection of caspase activation, mixed anterior horn cultures were double stained using specific antibodies against SMI32 and the activated form of caspase-3 and caspase-9. The quantification of SMI32-positive cells co-expressing the activated caspase-3 motor neurons in mixed anterior horn cultures, 6 hours after excitotoxic exposure, further confirmed the involvement of apoptosis in motor neurons treated with 5μM kainate and the selective anti-apoptotic role of EPO (Fig 33 A). On the other hand 1μM AMPA did not induce caspase-3 activation in SMI32-positive cells (Fig 33 A). Similarly to caspase-3, low AMPAR agonist concentration treatment increased the number of activated caspase-9 in SMI32 positive cells above control conditions and EPO reverted this effect (Fig 33 B). As expected, 1 μM AMPA treatment did not raise the mean number of activated caspase-9 positive motor neurons (Fig 33 B). The pattern of expression of either activated caspase-3 or activated caspase-9 in SMI 32-positive cells is shown in figure 33 C-E and figure 33 F-H respectively. The ratio between the percentage of activated caspase-3 positive cells and the percentage of activated caspase-9 positive cells, 6 hours after 5 μM kainate treatment, is about 3. Although this discrepancy may be due to a different sensitivity of antibodies used to reveal the two caspases, it is more likely that kainate-treated motor neurons did not have a synchronous activation of the apoptotic pathway. Therefore, it is possible that, 6 hours after kainate treatment, motor neurons which underwent early apoptotic activation already express both activated caspase-9 and caspase -3, whereas in motor neurons where apoptosis is slower only the cleaved form of caspase-3 can be detected.

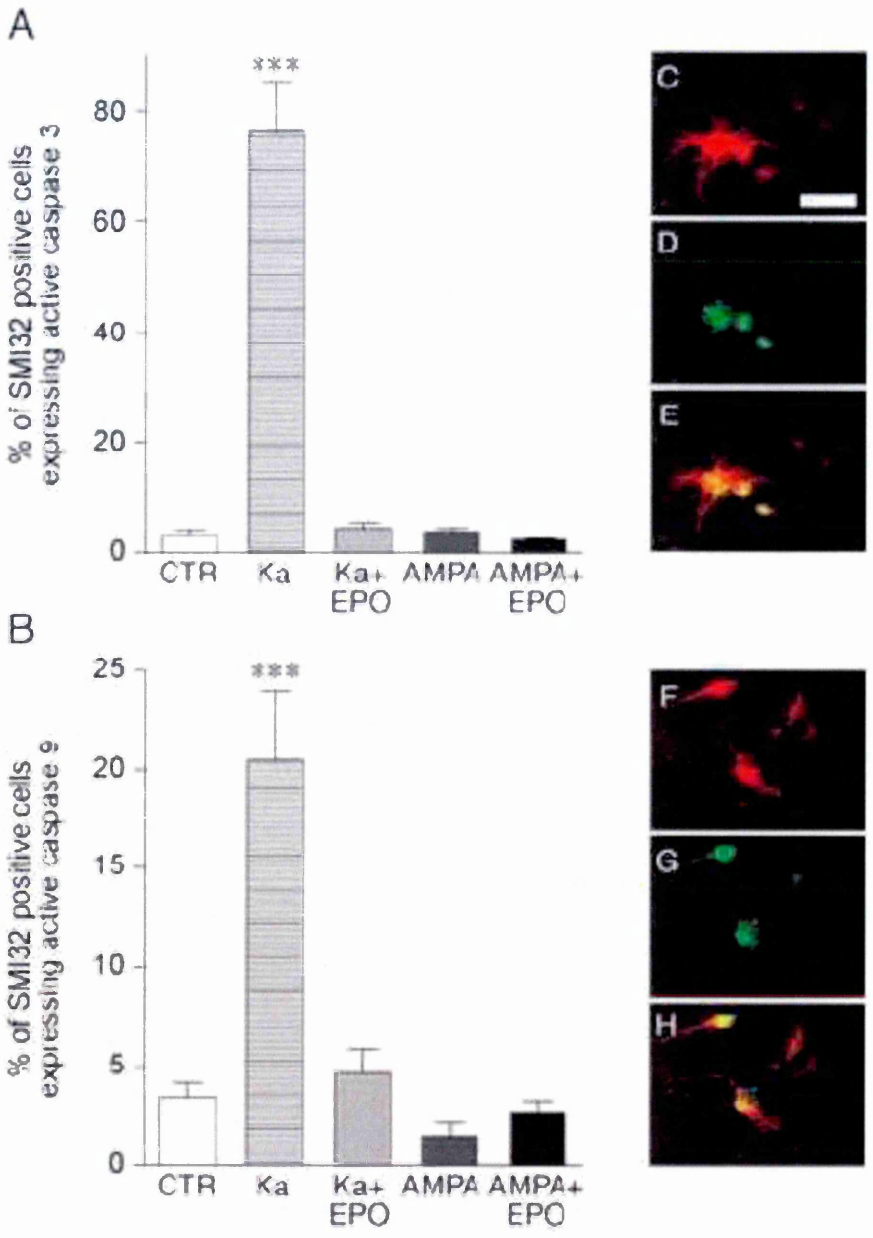


Figure 33. EPO antagonizes the caspase activation induced by low AMPAR agonist concentrations.

Mixed anterior horn cultures pre-treated with EPO (2.5 pmol/mL) for 72 h and then exposed for further 6 h to low (5 µM KA) or high (1 µM AMPA) AMPAR agonist concentrations (alone or in co-treatment with the cytokine) were double-stained by SMI32 and the antibody for activated capase-3 (A) or -9 (B). Histograms show the percentage of SMI32-positive cells displaying activation of caspase-3 (A) or -9 (B).

*** $p < 0.001$ vs control and the other treatment condition.

C-E: co-localization of activated caspase-3 (green, D) in SMI32 positve cells (red, C) after kainate treatment.

F-H: co-localization of activated caspase-9 (green, G) in SMI32 positve cells (red, F) after kainate treatment. Scale bar C-H, 40 µm.

To test the neuroprotective properties of EPO on the cell death triggered by a different toxic condition, we investigated the effect of the cytokine on motor neuron viability in purified motor neuron cultures after 18 hours of serum deprivation. Under these toxic conditions motor neuron viability was significantly reduced and EPO completely reversed this toxicity (% cell viability in control condition: 100.0 $\pm$ 7.8; in serum-deprived cells: 67.4 $\pm$ 4 *** $p < 0.001$; in serum-deprived cells $\pm$ EPO: 107.3 + 4.9 n.s.), confirming previous published results (Siren *et al.* 2001).

The percentage of apoptotic nuclei, as revealed by DNA staining with Hoechst 33258, was hugely increased compared to control values and 2.5 pmol/mL EPO significantly reduced the percentage of apoptotic nuclei in serum-deprived cultures (Fig 34 A). After the staining of purified motor neurons by the specific antibody, the number of motor neurons expressing activated caspase-3 was increased 6 hours after serum deprivation and was significantly reduced by EPO treatment (Fig 34 B). Figure 34 shows representative pictures of purified motor neuron cultures incubated with Hoechst 33258, panels C-E, and immunostained to detect the activated caspase-3, panels F-H. In control condition a high number of nuclei displayed physiological morphology (Fig 34 C). In addition a very low number of cells positive for the activated form of caspase-3 can be detected (Fig 34 F). Purified motor neurons showed a marked increase in fragmented nuclei after serum deprivation (Fig 34 D). In this condition the number of activated caspase-3 cells was also greatly increased (Fig 34 G). EPO alone did not produce any significant difference either in Hoechst 33258 binding or in activated caspase-3 immunostaining compared to control conditions (not shown). Representative pictures showing the effect of EPO in reducing nuclear fragmentation and caspase-3 activation after serum deprivation are in Fig 34, panels E and H respectively.

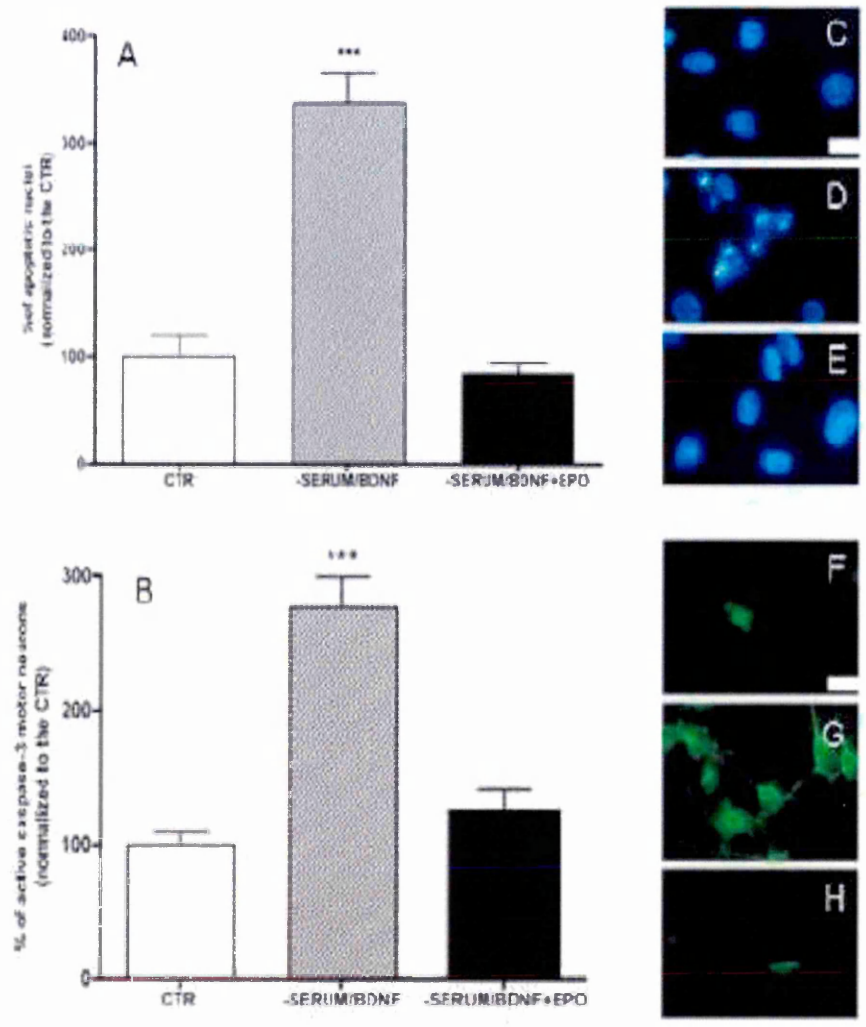


Figure 34. EPO antagonizes the nuclear fragmentation occurring after serum/growth factor deprivation.

Mixed anterior horn cultures where pre-treated (where present) with EPO (2.5 pmol/mL) for 72 h and then deprived of serum and growth factors for further 18 h were double-stained by SMI32 and Hoechst 33258 (A) or activated capase-3 (B). Histograms show the mean percentage ± SD (6 replicates from 3 independent experiments) of SMI32-positive cells displaying nuclear fragmentation (A) or activation of caspase-3 (B). *** $p < 0.001$ vs control and EPO treatment, ANOVA and Tukey test. C-E: Hoechst 33258 staining in purified motor neuron cultures. In basal conditions high percentage of nuclei shows good morphology (C); serum withdrawal drastically increases the nuclear fragmentation (D); EPO treatment restores the nuclear morphology (E).
F-H: activated caspase-3 immunostaining. In basal condition very few cells show activation of caspase-3 (F); serum deprivation increases the immunoreactivity for activated caspase-3 antibody (G); EPO treatment drastically reduces the number of active caspase-3 positive cells revealed in serum deprivation condition (H). Scale bar, C-E, 20 μm: F-H, 40 μm.

9.1.2 Effect of non-erythropoietic EPO derivatives

We extended the in vitro studies on motor neuron cultures to different non-erythropoietic EPO derivatives, i.e. ASIALO-EPO, CEPO and to the EPO fragment HBP. We initially verified if the CD131 receptor was expressed in mixed anterior horn cultures. Cells were double-stained with SMI32 and the specific antibody for CD131. CD131 showed a pattern of expression similar to that of EPOR, being both in motor neurons (Fig 30 F, merge staining) and in SMI32-negative cells (Fig 30 F, red) which were intensively stained by the antibody.

Under the same treatment schedule used for EPO (added to mixed anterior horn cultures 3 days before treatment and re-added with the glutamate agonist), both ASIALO-EPO and CEPO, tested at 100 ng/mL (equimolar concentration of EPO), significantly prevented the 5 μM kainate-induced motor neuron death (Table III).

To detect whether the EPO region that interact with CD131 was sufficient to induce neuroprotection, we tested the synthetic peptide HBP. HBP (100 ng/mL), used with the pre-treatment schedule, exerted potent neuroprotective activity comparable to EPO and to CEPO since it completely blocked the neurotoxic effect of 5 μM kainate (Table III).

Conclusions

We have shown here that EPO protects motor neurons from two different stimuli suggested to be responsible for human ALS: neurotrophic factors deficiency and excitotoxicity. Under the experimental conditions utilised in the present study, activation of the apoptotic pathway by serum deprivation or treatment with low kainate concentrations was clearly shown by DNA fragmentation and nuclear condensation, activation of caspase-3 and, mainly upon kainite exposure, caspase-9 cleavage. On the contrary, high AMPA concentration did not activate apoptotic motor neuron death (as shown in detail in Chapter 6). The neuroprotective effect of EPO was exerted

TABLE III. Effect of kainate treatment on motor neuron viability in the absence or presence of EPO derivatives.

% motor neuron viability after 5 μM kainate		
(No. replicates)	**Without EPO derivatives**	**With EPO derivatives**
Asialo-EPO (12)	58 ± 11	91 ± 12***
CEPO (15)	42 ± 12	67 ± 19 **
HBP (6)	57 ± 9	104 ± 12 ***

Viability of motor neurons in mixed neuron/glia cultures after 48 h of incubation with kainate (5 μM). When present, EPO derivatives (100 ng/mL) were added to motor neuron cultures 72 h before treatment and re-added with kainate. Data represent mean ± SD of SMI32-positive cells, normalized to control. For all EPO derivative treatment F_{int} $p < 0.001$.

*** $p < 0.001$, ** $p < 0.01$ vs. condition without EPO derivatives, Two-way ANOVA and Bonferroni post tests.

exclusively against the apoptotic motor neuron death through the inhibition of the intrinsic apoptotic pathway. This selective mechanism of protection has been characterized in our study either by evaluating the effect on cell survival or investigating the different features of cell death in all experimental conditions we utilized. In addition, we reported that in basal conditions, the 5 day-treatment with EPO produced a neurotrophic effect, increasing the neurite outgrowth and the number on SMI32 positive motor neurons in mixed anterior horn cultures. A similar effect was obtained in purified motor neurons (Siren *et al.* 2001) and it is likely related to a decrease in spontaneous apoptosis. The reduction below control values of the percentage of apoptotic nuclei and of activated caspase-3-positive cells further support this hypothesis. However, EPO treatment did not produce significant protection when motor neurons were exposed to a high concentration of AMPA that triggers a non-apoptotic cell death pathway. Our data demonstrate EPO protection against low AMPAR agonist concentration but not the higher concentrations in cultured motor neurons. These discrepancies could be related to the type of death induced by excitotoxins, depending on their concentration.

The in vivo utilization of chronic EPO treatment may be limited by modification of receptor expression and by the specific death pathway followed by neurodegeneration. However, the most important limitation is due to EPO activity on the hematocrit that could produce serious side-effect in patients. Thus, different non-erythropoietic molecules retaining the neuroprotective activities of EPO have been derived from EPO. We tested here two non-erythropoietic EPO derivatives, CEPO and ASIALO-EPO and a synthetic CD131 binding fragment of the protein, HBP, on AMPAR-mediated toxicity. They all exerted a potent neuroprotective effect on primary cultured motor neurons following stimulation of AMPA receptors by low concentrations of kainate.

These results indicate that such molecules, devoid of hematopoietic activity, could exert tissue-protective effects similar to EPO, thus offering a potential pharmacological approach to the pathology.

9.2 Reparixin

As previously demonstrated (Chapter 7.2) CXCR2 activation induces direct toxicity on motor neurons. The finding of increased IL-8 levels in the CSF of ALS patients (23±9 pmol/mL in ALS vs. 16±5 pmol/mL in controls, $p = 0.0026$; CSF obtained by 38 ALS patients or 18 subjects with non-inflammatory neurological diseases; De Paola *et al.*, in preparation) suggests that CXCR2-mediated neurotoxicity may represent an important contribution to a disease mainly characterized by motor neuron degeneration. Thus, the development of pharmacological strategies aimed at the limitation or inhibition of the CXCR2-mediated effect would represent a major advancement for the prevention of neuronal death in this toxic condition.

Reparixin, is a new small-molecule inhibitor of human CXCR1/R2 and rat CXCR2 receptor activation. Structural and biochemical data are consistent with a non-competitive allosteric mode of interaction between CXCL8 receptors and reparixin L-lysine salt, which by blocking CXCR1 and CXCR2 in an inactive conformation prevents the activated receptor-induced intracellular signal transduction cascade and cell response. We tested here reparixin (as L-lysine salt) efficacy on motor neuron death induced by MIP-2 (see Chapter 7, section 7.2) and verified that it specifically exerted its effect by inhibiting CXCR2.

9.2.1 Neuroprotective effect of reparixin

Mixed anterior horn cultures or purified motor neuron cultures were exposed to MIP-2 at a concentration previously demonstrated to induce neurotoxicity (12.5 nM, see

Chapter 7, section 7.2.2) for 48 hours. When indicated, co-treatments were performed by simultaneously adding the reparixin solution. Figure 35 shows that reparixin (1–10 μM in mixed cultures) counteracted the toxicity of 12.5 nM MIP-2, both in mixed (Fig 35 A) and purified (Fig 35 B) rat motor neuron cultures, with an IC 50 of 1 μM . To demonstrate that the protective effect of reparixin specifically involved CXCR1/2, we also tested DF1726A, which is structurally related to reparixin but is not active on IL-8 chemotaxis in human PMN at a wide range of concentrations (10^{-5} –10^{-8} M; Bertini *et al.*, unpublished data). DF1726A, under the same conditions used for reparixin in mixed cultures (10 μM for 48 h), did not protect motor neuron cultures from the toxicity of 12.5 nM MIP-2 (Fig 35 A). To support the specific neuroprotective action of reparixin against the CXCR2-mediated toxicity, we tested its effect on AMPA receptor-mediated motor neuron death. Under the experimental conditions used (48 h, 5 μM), kainate induced about 50% death in mixed anterior horn cultures and this was not affected by 10 μM reparixin (Fig 36). Similarly, in purified motor neurons, reparixin did not counteract the effect of 50 μM kainate (45 $\pm$ 4 and 52 $\pm$ 1% kainate-induced death in the vehicle- and reparixin-treated groups, respectively).

Conclusions

We demonstrated here for the first time that reparixin specifically prevented the MIP-2-induced death of motor neurons in vitro. This effect is not mediated by glial cells since reparixin was neuroprotective also in purified motor neuron cultures. This finding supports the encouraging results from the literature showing reparixin-dependent neuroprotection also in *in vivo* models (Gorio *et al.* 2007, Villa *et al.* 2007) and suggests that this drug could exert important pharmacological effects in addition to its anti-inflammatory properties.

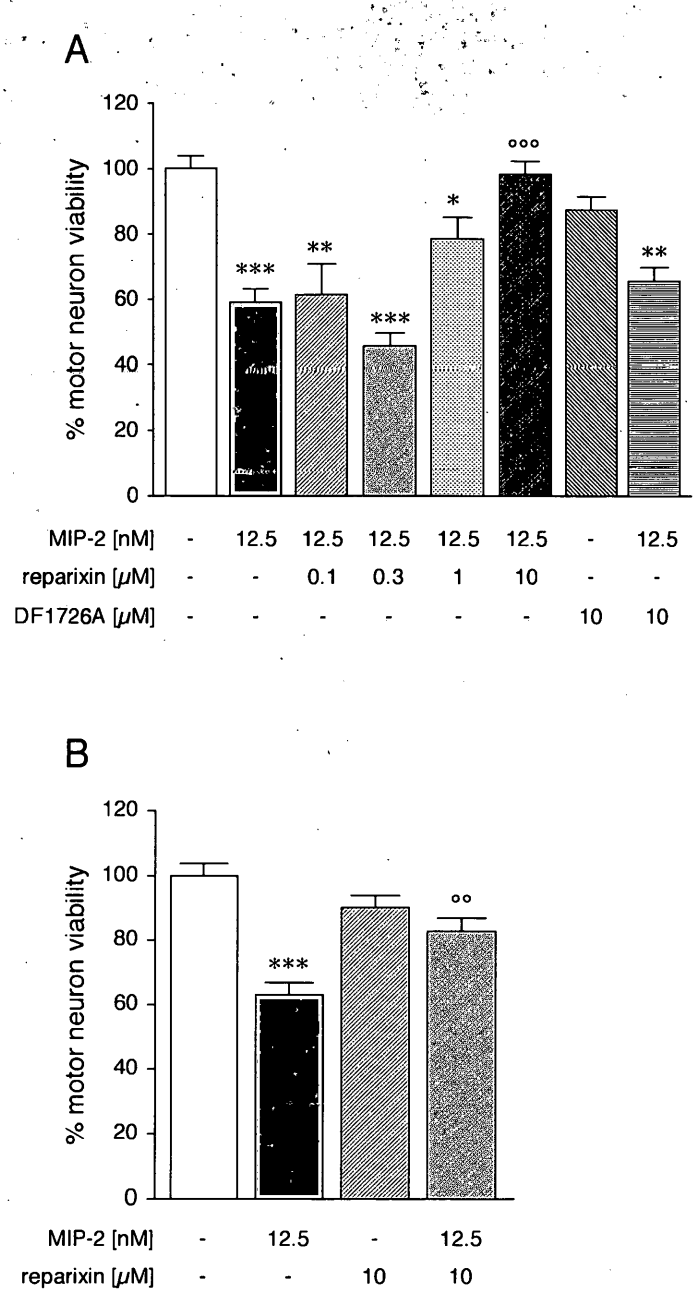


Figure 35. Reparixin is neuroprotective against MIP-2-induced toxicity in both mixed and purified motor neuron cultures.

Mixed anterior horn cultures (A) or purified motor neurons (B) were exposed for 48 h to a toxic concentration (12.5 n M) of MIP-2 alone or with different concentrations of reparixin (100 nM to 10 μM).

The reparixin analogue DF1726A was also tested in mixed cultures (A).

Data are expressed as the mean percentage ± SD of 6 replicates from 3 independent experiments.

* $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$ vs control; †† $p < 0.01$, ††† $p < 0.001$ vs 12.5 nM MIP-2 alone, ANOVA and Tukey test.

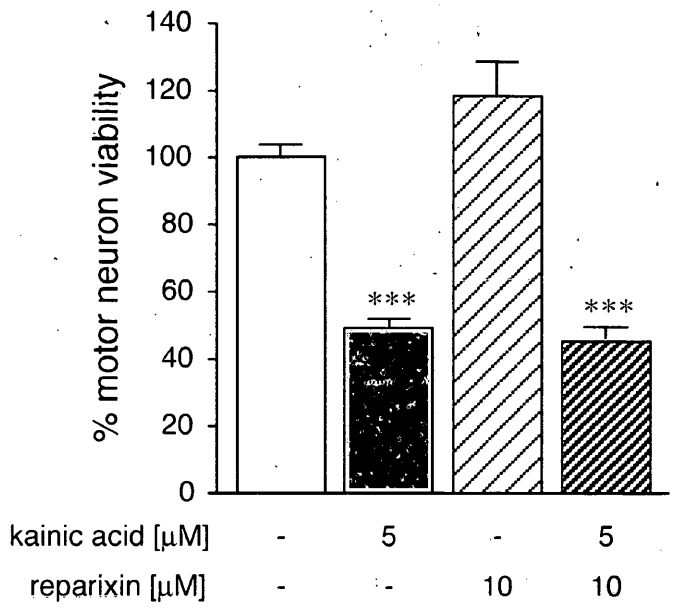


Figure 36. Reparixin does not prevent the kainate-induced motor neuron death in mixed anterior horn cultures.

Mixed anterior horn cultures were exposed for 48 h to 5 µM kainate alone or with 10 µM reparixin. Data are expressed as the percentage of SMI32-positive cells normalized over controls, and are means ± SD of 8 replicates from 4 independent experiments. *** $p < 0.001$ different from control and reparixin alone, ANOVA and Tukey test.

DISCUSSION

The most striking evidence when facing ALS is the lack of effective or at least encouraging treatments for fighting the disease. Interventions aimed at prolonging the patient's survival by a few months or palliative care applications represent an attempt to patch up the problem rather than to offer real solutions. The deficiency of valid pharmacological therapies is the consequence of the failures in elucidating the triggering events underlying the pathology and of the still incomplete information coming from the use of animal models. Most of the pharmacological studies are performed *in vivo* on the SOD1 transgenic mouse that, although being a valid model of the familiar form of ALS, brings mutations representing only 2% of the ALS cases in humans and is anyway poorly functional for pharmacokinetic studies. Moreover, the latest epidemiological studies describe ALS as a disease in continuous evolution, showing new features in terms of disease progression, course and incidence when compared to data from the previous decade (Beghi *et al.* 2007). These are clearly related to the evolution of human life-style. ALS is currently considered to be an age-related disease, with cigarette smoking being the only risk factor supported by fairly good epidemiological evidence (Beghi *et al.* 2007). New clusters of people with increased ALS incidence have been discovered, different from those already known, to introduce new unknown, possibly environmental factors for the onset of ALS. All this evidence makes the therapeutic solution even more difficult to be successful. Thus, parallel *in vitro* and *in vivo* studies on both patient's specimen and animal models are required in order to discover the mechanisms activated in motor neurons under such peculiar toxic conditions. Among all the different *in vitro* models, primary cultures have been the most used over the years and have provided a huge amount of valid and useful information on the physiological and pathological role of the neural population.

DISCUSSION

In the present study primary motor neuron cultures were used to investigate the role of AMPAR-dependent excitotoxicity in motor neuron death. The effects of TNF-α and IL-8 were also considered in order to study the interaction between the neuroinflammatory response and the excitotoxic damage. Finally we evaluated the role of protein aggregation by analyzing the effect of α-synuclein accumulation in motor neurons. In order to propose valid pharmacological approaches for this pathological condition, different compounds interacting with the considered death pathways were tested.

Excitotoxicity

Glutamate-induced excitotoxicity is the best-characterized factor in the pathogenesis of ALS and AMPA/kainate receptors mainly mediate calcium-dependent motor neuron death in such toxic conditions (Carriedo *et al.* 2000, Carriedo *et al.* 1996). We reported here a successful approach to the in vitro study of AMPAR-mediated intracellular events occurring in motor neurons when exposed to different excitotoxic stimuli. We set up and standardized an easy and quick model of cocultures of purified motor neurons and glial cells which showed significant improvement in motor neuron health and survival compared to other previously reported methods. This allowed a detailed study of the AMPAR-mediated neurodegenerative mechanisms as well as other important events related to the AMPAR-dependent pathways, which occur in ALS. The main objective of this study was to fully analyze the effect of AMPAR agonists on the main events activated during apoptotic cell death, i.e. mitochondrial cytochrome *c* release, activation of the caspase cascade, externalization of the phosphatidylserine residues on the outer membrane surface and DNA fragmentation. This cell death pathway appears to occur in both ALS animal models and patients (Cleveland & Rothstein 2001, Friedlander 2003, Pasinelli *et al.* 2000). The expression of the anti-apoptotic protein Bcl-2 is decreased, whereas that of the pro-apoptotic protein Bax is increased in the

spinal cord of ALS patients and of transgenic mSOD1 mice (Martin 1999, Vukosavic *et al.* 1999). After a death stimulus, cytosolic Bax translocates to mitochondria (Gross *et al.* 1998, Wolter *et al.* 1997) where it can promote the release of cytochrome *c*. Immunocytochemical evidence of cytochrome *c* translocation in motor neurons of spinal cords from sALS patients or from transgenic mSOD1 mice has been reported and it occurs in parallel with the neurodegenerative process in the animal model (Guegan *et al.* 2001). Activation of caspases is likely to participate in the neurodegenerative process of ALS, since caspase-1, -3, -7 and -9 are activated in spinal cords from affected transgenic mSOD1 mice (Guegan *et al.* 2001, Pasinelli *et al.* 1998, Pasinelli *et al.* 2000, Vukosavic *et al.* 2000).

A dual role of glutamate in triggering either necrosis or apoptosis with time and concentration dependence has been reported in cerebellar granule cell cultures (Ankarcrona *et al.* 1995). Consistently, here we clearly demonstrated that, also in the motor neuron, the main cellular target of ALS, AMPAR agonists can induce different intracellular death pathways depending on the intensity of the initial stimulus.

In fact, when low AMPAR agonist concentrations (inducing less than 25% motor neuron death within 6 hours) were used, we could observe activation of the apoptotic machinery in motor neurons, starting from the earlier events such as the mitochondrial cytochrome *c* release and the following activation of the caspase cascade, up to the latest, including chromatin condensation and DNA fragmentation externalization and re-arrangement of phosphatidylserine composition of the outer membrane. On the other hand, higher concentrations, triggering more than 35% death after 6 hour treatment, do not induce any significant effect on the different apoptotic events considered in the study, compared to control conditions.

A key triggering factor, dependent on the intensity of the initial stimulus to the AMPAR and able to lead to the activation of different patterns of death, is the amount of

intracellular calcium influx and resulting compromised calcium homeostasis. When Ca^{2+} influx is slow and relatively moderate in quantity the pathway involving protease cleavage is activated. On the other hand, stronger glutamatergic stimulation can produce a massive Ca^{2+} influx which reduces the functionality of different organelles such as mitochondria and the endoplasmic reticulum thus provoking such a fast decay in cellular activity that the cells, which might be triggered to undergo apoptosis, are instead forced to die by necrosis (Volbracht *et al.* 1999). The role of $[Ca2^{+}]_i$ in AMPAR-mediated excitotoxicity was studied *in vitro* by exposing neurons to high AMPAR agonist concentrations rapidly triggering neuron death (Arundine & Tymianski 2003, Carriedo *et al.* 2000, Van Damme *et al.* 2003, Van Den Bosch *et al.* 2000, Vandenberghe *et al.* 2000). Motor neurons were more sensitive to Ca^{2+} - dependent degeneration after exposure to kainate or AMPA than other neurons (Arundine & Tymianski 2003, Carriedo *et al.* 2000, Van Damme *et al.* 2003, Van Den Bosch *et al.* 2000, Vandenberghe *et al.* 2000). Kainate or AMPA induced a selective rise in cytoplasmic Ca^{2+} concentration, reactive oxygen species and mitochondrial Ca^{2+} overload in motor neurons (Carriedo *et al.* 1996, Sen *et al.* 2008, Van Den Bosch *et al.* 2000), suggesting that mitochondria are one of the main targets of AMPAR-dependent calcium influx. Recently, mitochondrial failure in buffering the kainate-dependent persistent increase of cytosolic calcium was reported and this was partially attributed to the lower density of such organelles in motor neurons (Grosskreutz *et al.* 2007).

Here we detected the $[Ca^{2+}]_i$ variations induced by different AMPAR agonist concentrations able to activate specific intracellular pathways leading to motor neuron death. We revealed that higher AMPAR agonist concentrations induce higher AMPAR-dependent intracellular calcium influx compared to lower concentrations, thus causing a severe alteration in calcium homeostasis and possible rapid non-apoptotic degeneration. By contrast, lower AMPAR agonist concentrations trigger a milder increase in $[Ca^{2+}]_i$

which could allow the cell to activate secondary intracellular events and the complex apoptotic machinery under persistent toxic conditions.

Pharmacological treatments with EPO were performed against AMPAR-dependent motor neuron death since this compound has shown interesting neuroprotective properties in different models of neurodegenerative disease (Agnello *et al.* 2002, Bianchi *et al.* 2004, Savino *et al.* 2006, Siren *et al.* 2001, Viviani *et al.* 2005, Wang *et al.* 2004).

We showed here that EPO treatment protects motor neurons from two different stimuli suggested to be responsible in human ALS: neurotrophic factors withdrawal and excitotoxicity. However, the neuroprotective effect of EPO was exclusively exerted against the apoptotic motor neuron death occurring after serum deprivation or treatment with low AMPAR agonist concentrations through the inhibition of the intrinsic apoptotic pathway. This selective mechanism of protection has been characterized in our study either by evaluating the effect on cell survival or investigating the different features of cell death in all experimental conditions we utilized. In addition, we reported that in the basal condition, the repeated treatment with EPO produced a neurotrophic effect, increasing the neurite outgrowth and the number on SMI32-positive motor neurons in mixed anterior horn cultures. A similar effect was reported in the literature also in purified motor neurons (Siren *et al.* 2001) and it is likely related to a decrease in spontaneous apoptosis rather than to an increase in the number of new cells. This is consistent with other reports that EPO does not stimulate cell proliferation in neuronal cultures (Siren *et al.* 2001). We showed here reduction below control values of the percentage of apoptotic nuclei and of activated caspase-3 positive cells upon EPO treatment which lends further support for this hypothesis. However, we did not detect any significant protection by EPO when motor neurons were exposed to a higher concentration of AMPA that triggers non-apoptotic cell death . The effect of EPO on

excitotoxicity in cellular models in vitro is somewhat confusing: protection against glutamate exposure was reported in cultured hippocampal and cortical neurons (Morishita *et al.* 1997) and in cortical neurons exposed to NMDA (Bernaudin *et al.* 1999). Conversely, it has also been reported that EPO protects cortical neurons from the toxicity induced by AMPA, but not by glutamate or NMDA (Sinor & Greenberg 2000). These discrepancies could be related to the type of death induced by the different concentrations. Along those lines, our present data report EPO protection against low, but not high, AMPAR agonist concentrations indicating the specific prevention of apoptotic motor neuron death .

EPO has been tested in vivo and found to be active on acute spinal cord injuries, such as ischemia (Celik *et al.* 2002) or trauma (Gorio *et al.* 2002) where excitotoxicity plays an important role. However, studies aimed at testing the effect of EPO in the mSOD1G93A mouse model reported controversial results. In 2007, Koh and colleagues showed that EPO was protective in this model in that it delayed symptom onset, rotarod failure and endpoint, and also by prolonging the symptom duration. Its protective effects might be achieved through inhibition of both motor neuron death and inflammation (Koh *et al.*, 2007). Noteworthy, in the same year Grunfeld et al. found a modest delay in disease onset in female mice only, without an effect on survival after EPO treatment. The hematocrit rose to a similar extent in female and male treated mice supporting the authors' conclusion that the effect of EPO on motor function was sex specific and not secondary to an unequal effect on hematocrit (Grunfeld *et al.*, 2007). Firm conclusions on whether EPO was neuroprotective in this mouse model were not possible from that study since the study sample size was small and the mice were unequally distributed between the treatment and control groups (20 and 14 respectively).

Another group of investigators published that EPO did not preserve motor neurons in the mSOD1G93A animal model of ALS (Grignaschi *et al.*, 2007). There were several

methodological differences between the two EPO studies. These include use of different forms and dosages of EPO and different routes of administration: subcutaneous versus intraperitoneal. While treatment was initiated at approximately the same age in both studies, Grignaschi et al. utilized a slight variation in rotarod task, administered treatment more frequently, and also measured hematocrit more frequently. Treatment by Grignaschi et al. significantly increased mice hematocrit compared to Grunfeld et al., yet finding no preservation of spinal cord motor neurons and no sex-specific effects. Both groups report a delay in the decline of rotarod performance. Grignaschi et al. conclude that increased oxygen delivery to muscles may be responsible for this effect on rotarod performance. Neither group reported an effect on survival.

The effect of chronic EPO treatment may be limited by modification of receptor expression and by the specific death pathway followed by neurodegeneration. However, the most important limitation is due to EPO activity on hematocrit that could produce serious side-effects in patients. Thus, we also successfully tested different non-erythropoietic EPO derivatives that could be more suitable for chronic therapeutic strategies. ASIALO-EPO, CEPO and the synthetic CD131-binding-fragment HBP all showed potent anti-apoptotic properties against AMPAR-dependent motor neuron death similar to those observed for EPO. The in vivo protective effect of CEPO has already been described for spinal cord compression, diabetic neuropathy, and experimental autoimmune encephalomyelitis (Leist *et al.* 2004). Furthermore, we found that CEPO, and, to a lesser extent, ASIALOEPO, could exert neuroprotective effects in a model of chronic motor neuron degeneration (the wobbler mouse) and reduce inflammation in the anterior horn of the spinal cord without increasing hematocrit levels (Mennini *et al.* 2006).

HBP is small peptide and has proteolytic features which, presumably, confer a very short plasma half-life to the peptide. Nevertheless HBP has been found to be protective

in a rat model of middle cerebral artery occlusion in which EPO (Brines *et al.* 2000), ASIALO-EPO (Erbayraktar *et al.* 2003), and CEPO (Leist *et al.* 2004) have previously been shown to produce strong protective effects. Recently, the tissue protective effect of HBP has also been demonstrated *in vivo* in a variety of other models, including ischemic stroke, diabetes-induced retinal edema, and peripheral nerve trauma (Brines *et al.* 2008). The results of these experiments showed that the tissue-protective activities of EPO are mimicked by small, non-erythropoietic peptides that recapitulate a portion of EPO's three-dimensional structure acting on CD131, thus suggesting this receptor and related pathways as possible therapeutic targets.

Neuroinflammatory mediators

Inflammatory reactions have been implicated in several pathogenic mechanisms of motor neuron diseases, including ALS (Appel *et al.* 1995, Cereda *et al.* 2008, Poloni *et al.* 2000, Yi *et al.* 2000). In particular, high concentrations of IL-6, TNF and MCP-1 were detected in the cerebrospinal fluid or plasma of ALS patients, suggesting a neuroinflammatory component (Baron *et al.* 2005, Cereda *et al.* 2008, Ford & Rowe 2004, Gallo *et al.* 1994, Joerg Stuerenburg *et al.* 1999, Krieger *et al.* 1992, Moreau *et al.* 2005, Poloni *et al.* 2000, Sekizawa *et al.* 1998, Wilms *et al.* 2001). Thus, our investigation about possible interactions between the effect of important mediators of the inflammatory response, such as TNF-α and IL-8, and the excitotoxic injury in motor neurons could be of great relevance for the understanding of such ALS pathogenic mechanisms.

TNF-α

Previous findings reported a dual effect of TNF-α on excitotoxic neuronal damage. TNF-α had a neuroprotective effect against AMPAR-mediated excitotoxicity in organotypic hippocampal slice cultures (Bernardino *et al.* 2005) and showed anticonvulsant effects against kainate-induced seizures (Balosso *et al.* 2005). Furthermore it induced a neuroprotective effect against excitotoxic insults by promoting the maintenance of calcium homeostasis (Cheng *et al.* 1994) or the activation of the transcription factor NF-kB (Marchetti *et al.* 2004). On the other hand it strengthened glutamatergic synaptic transmission by increasing the surface expression of AMPA receptors (Beattie *et al.* 2002, Stellwagen *et al.* 2005) and human neuronal cell lines showed increased susceptibility to kainate after TNF-α treatment. Furthermore, the combination of glutamate and TNF-α provoked an amplified neurotoxic effect mediated by the AMPAR in the rat spinal cord (Hermann *et al.* 2001).

We reported here that TNF-α treatment induced motor neuron death only in cocultures, where a mature glial cell layer was present. We also documented a significant interaction between the effect mediated by TNF-α and the AMPAR-dependent toxicity in cocultures, while no effect was revealed in mixed anterior horn cultures. This evidence demonstrates that the *in vitro* aging of the glial population is a determinant for eliciting TNF-α-mediated neurotoxicity and protection against the AMPAR-mediated motor neuron death. Many authors have associated the dual effect of TNF-α with its interaction with its two different receptors. In particular, using mice lacking TNF-α receptors or selective stimulation of TNFR1 by human TNF-α, Bernardino and colleagues (Bernardino *et al.* 2005) defined the selective involvement of TNF-α receptor subtypes in the opposite effects of the cytokine on AMPA-induced cell death. Namely, that the neuroprotective effect was mediated by TNFR2, whereas TNFR1

mediated exacerbation of the AMPA toxicity. In accordance, TNFR1 has been implicated in cell death by a variety of authors (Fontaine *et al.* 2002, Stellwagen *et al.* 2005, Yang *et al.* 2002), while TNFR2 was suggested to mediate neuronal survival (Balosso *et al.* 2005, Fontaine *et al.* 2002, Marchetti *et al.* 2004). Thus, the mechanisms modulating the expression of these two receptors and the subsequent intracellular signal(s) may determine neuronal responsiveness to TNF-α and its interaction with excitotoxicity.

We frequently revealed high immunoreactivity of TNF receptors in 4 week old glial cells (used in cocultures), while we could not document the presence of the receptors on glial cells grown in vitro for 1 week (mixed anterior horn cultures), suggesting that both the neuroprotection and the neurotoxic effect induced by TNF-α on motor neurons are dependent on the functional expression of its receptors.

IL-8

Chemokines were reported to have direct effects on neurons. In particular, the IL-8 receptor CXCR2 is expressed in neurons and may play a pathophysiological role in neurodegenerative diseases, like Alzheimer disease (Horuk *et al.*, 1997; Xia *et al.*, 2002). In the present study we aimed at verifying the role of CXCR2 on motor neuron degeneration. The few data that have been published on the trophic or toxic effects of CXCR1/2 and their ligands were obtained from experiments on neuronal cultures distinct from motor neurons, and they are controversial. IL-8 enhanced the survival of hippocampal cultures in vitro, possibly by an indirect effect mediated by increased astroglial and microglial proliferation (Araujo *et al.*, 1993). IL-8 also showed neuroprotective activity against NMDA or β-amyloid-induced toxicity in mixed cortical cultures (Bruno *et al.*, 2000). A protective effect of MIP-2 (CXCR2 agonist in rodent) against apoptosis induced by low potassium-containing medium was reported in

cerebellar granule cells (Limatola *et al.*, 2000, 2002). However, other reports found that IL-8 has detrimental effects such as induction of Tau phosphorylation (Xia *et al.*, 2002) and of pro-apoptotic proteins in primary neurons (Thirumangalakudi *et al.*, 2007). This is the first report of a toxic effect of a CXCR2 ligand on motor neurons. We found that MIP-2 induced dose-dependent neurotoxicity in mixed anterior horn cultures and purified motor neuron cultures, indicating a direct toxic effect of CXCR2 activation on the motor neuron, without the involvement of glial cells. We also confirmed the presence of CXCR2 receptors on motor neurons, but could not document specific immunostaining for CXCR1, which is in agreement with earlier studies showing that CXCR2, but not CXCR1, is expressed at high levels in certain CNS regions including motor neurons in the anterior horn of the human spinal cord (Horuk *et al.*, 1997). The lack of effect of MIP-2 on mixed anterior horn cultures of CXCR2-deficient mice further indicated that the toxicity is specifically mediated by this receptor. The present finding of direct neurotoxicity induced by CXCR2 activation on motor neurons suggests new pathogenic mechanisms for ALS. The direct role of CXCR2 ligands in neuronal death *in vivo* is indicated by the fact that intrahippocampal injection of MIP-2 results in the apoptotic cell death of hippocampal neurons (Kalehua *et al.* 2004).

Preliminary studies on the spinal cord of wobbler mice indicated high levels of CXCR2 ligands (De Paola, in preparation), further supporting a possible role of this chemokine receptor in vivo. These results not only add a new piece to the picture of the complex relationships between inflammatory molecules and CNS diseases, but also open the way to new therapeutic avenues.

We reported a possible pharmacological approach to the prevention of CXCR2-mediated neurotoxicity by testing the effect of reparixin, an orally active CXCR1/2 inhibitor which has been shown to reduce PMN infiltration and have protective activity in rat models of cerebral ischemia (Garau *et al.*, 2005; Villa *et al.*, 2007). It is currently

being tested in a phase-2 clinical trial for graft dysfunction after kidney or lung transplantation. We found that reparixin specifically prevented the MIP-2-induced death of motor neurons in vitro. The finding by Gorio *et al.* (Gorio *et al.*, 2007) of the efficacy of reparixin in reducing MIP-2 concentrations, oligodendrocyte apoptosis and axon demyelination in an in vivo model of spinal cord injury supports the possible neuroprotective role of CXCR2 inhibitors in the spinal cord. These results may be relevant not only for ALS, where chemokines released from activated glial cells can contribute to motor neuron degeneration, but also for other diseases associated with a secondary loss of motor neurons, including spinal cord injury and multiple sclerosis.

Protein aggregation

We also performed initial attempts to elucidate the effect of α-synuclein accumulation in cultured motor neurons, since protein accumulation represents an important event of ALS aetiology. The discovery of a-synuclein in Lewy bodies in PD (Polymeropoulos *et al.* 1997, Spillantini *et al.* 1997) was followed quickly by its detection in cellular inclusions in several other neurodegenerative diseases including cortical Lewy body dementia (Baba *et al.* 1998, Spillantini *et al.* 1998), multiple system atrophy (Arima *et al.* 1998, Fujiwara *et al.* 2002, Gai *et al.* 1999, Tu *et al.* 1998, Wakabayashi *et al.* 1998a, Wakabayashi *et al.* 1998b), Hallervorden-Spatz syndrome, now called neurodegeneration with brain iron accumulation type 1 (Arawaka *et al.* 1998, Saito *et al.* 2000, Wakabayashi *et al.* 1999), and amyotrophic lateral sclerosis (Mezey *et al.* 1998). Aggregates of α-synuclein have been observed in neuronal spheroids, astrocytes, Schwann cells and in cortico-spinal axon tract fibers and glia in brain and spinal cord of ALS patients (Doherty *et al.* 2004, Mezey *et al.* 1998). In addition an increased expression of α-synuclein has been detected in the anterior horn in the spinal cord of SODG93A transgenic mice, an animal model of ALS (Chung *et al.* 2003).

Our experiments provided evidence of a dual neuroprotective/neurotoxic effect of α-synuclein in cultured motor neurons which is concentration-dependent. α-Synuclein, targeted into the motor neuron by the HIV1 protein TAT, exerted a neuroprotective effect against oxidative stress, but not AMPAR-dependent excitotoxicity or serum deprivation, at nanomolar concentrations. Conversely, at micromolar concentrations, α-synuclein induced motor neuron death, likely caused by intracellular protein aggregates, as revealed by the thioflavin-T assay. These results are in accordance with what has been reported in other cell cultures (Albani *et al.* 2004, Seo *et al.* 2002).

Conclusions

In fulfilment of the objectives of this research project we have provided a wide range of information on in vitro motor neuron degeneration. In an earlier phase we set up and established an easy and reproducible new method for the culture of motor neurons and glial cells which allowed the study of the single cell type as well as the interactions between the two neural populations. We then reported consistent insights into the activation of different intracellular mechanisms when motor neurons were exposed to some of the main toxic conditions that are relevant in ALS aetiology, i. e. excitotoxicity, oxidative stress, serum deprivation, neuroinflammation and protein aggregation. These data add a new understanding within the complex network of events occurring in affected motor neurons. Furthermore this *in vitro* model allows to obtain further information about the fine pathways activated in degenerating motor neurons under different toxic conditions. Even if already important by themselves such basic results about the mechanisms used by motor neurons to die have acquired a greater relevance to our understanding of how cell death might be prevented. In fact, to complete the research outcomes we successfully tested different compounds which could prevent or counteract motor neuron death, induced by toxic agents, by interfering with the

intracellular death pathways that we previously elucidated. Such pharmacological approaches are well supported by results from in vivo studies on animal models and suggest potential therapeutic applications for the treatment of ALS.

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Appendix I - Main external contribution to the work of thesis

Part of the thesis experiments availed of the important collaboration of external colleagues and researchers.

- Studies on calcium imaging by confocal microscopy were performed in collaboration with Prof. Daniela Curti and Dr. Farncesca Botti, Department of Molecular and Cellular Physiological and Pharmacological Sciences, University of Pavia, Italy and with the technical facilities of the University of Pavia.

- CXCR2-deficient mice were provided by Dompè, Dompé Research Centre, Dompé pha.r.ma s.p.a., and embryo genotyping was performed in collaboration with the Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy.

Appendix II - Publications arisen from the thesis material

Mennini T, **De Paola M**, Bigini P, Mastrotto C, Fumagalli E, Barbera S, Mengozzi M, Viviani B, Corsini E, Marinovich M, Torup L, Van Beek J, Leist M, Brines M, Cerami A, Ghezzi P. Nonhematopoietic erythropoietin derivatives prevent motoneuron degeneration in vitro and in vivo. *Mol Med. 2006 Jul-Aug;12(7-8):153-60.*

Bigini P, **De Paola M**, Pasquali C, Botti F, Curti D, Ghezzi P, Mennini T Erythropoietin protects primary motor neuron cultures from apoptotic but not necrotic death in vitro. *IJNN. 2007;3 (3): 201-207*

Massimiliano De Paola, Pasquale Buanne, Leda Biordi, Riccardo Bertini, Pietro Ghezzi and Tiziana Mennini. Chemokine MIP-2/CXCL2, acting on CXCR2, induces motor neuron death in primary cultures. *Neuroimmunomodulation 2007;14:310–316. DOI: 10.1159/000123834*

Massimiliano De Paola, Valentina Diana, Paolo Bigini and Tiziana Mennini. Morphological features and responses to AMPA receptor-mediated excitotoxicity of mouse motor neurons: comparison in purified, mixed anterior horn or motor neuron/glia cocultures. *Journal of Neuroscience Methods 170 (2008) 85–95. DOI:10.1016/j.jneumeth.2007.12.022*

Michael Brines, Nimesh S. A. Patel, Pia Villa, Courtenay Brines, Tiziana Mennini, **Massimiliano De Paola**, Zubeyde Erbayraktar, Serhat Erbayraktar, Bruno Sepodes, Christoph Thiemermann, Pietro Ghezzi, Michael Yamin, Carla C. Hand, Qiao-wen Xie, Thomas Coleman, and Anthony Cerami. Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. *PNAS August 5, 2008 vol. 105 no. 31: 10925-10930. DOI: 10.1073/pnas.0805594105*